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**BOD1L IS REQUIRED TO SUPPRESS DELETERIOUS RESECTION OF STRESSED
REPLICATION FORKS**

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23 **SUMMARY**

24 Recognition and repair of damaged replication forks is essential to maintain genome
25 stability, and is coordinated by the combined action of the Fanconi Anaemia and homologous
26 recombination pathways. These pathways are vital to protect stalled replication forks from
27 uncontrolled nucleolytic activity, which otherwise causes irreparable genomic damage. Here we
28 identify BOD1L as a component of this fork protection pathway, which safeguards genome stability
29 after replication stress. Loss of BOD1L confers exquisite cellular sensitivity to replication stress
30 and uncontrolled resection of damaged replication forks, due to a failure to stabilise Rad51 at
31 these forks. Blocking DNA2-dependent resection, or down regulation of the helicases BLM and
32 Fbh1, suppresses both catastrophic fork processing and the accumulation of chromosomal
33 damage in BOD1L-deficient cells. Thus, our work implicates BOD1L as a critical regulator of
34 genome integrity that restrains nucleolytic degradation of damaged replication forks.

35 INTRODUCTION

36 Replication stress is any pathological process that compromises the fidelity of genome
37 duplication (Zeman and Cimprich, 2014). The Fanconi Anaemia (FA)/homologous recombination
38 (HR) pathway plays a central role in combatting replication stress (Gari and Constantinou, 2009).
39 FA is a rare chromosomal instability syndrome characterized by severe developmental
40 abnormalities, tumour predisposition and a hypersensitivity to agents that induce DNA inter-strand
41 cross-links (ICLs), such as mitomycin C (MMC) and cisplatin. To date, mutations in at least 16
42 different genes (FA complementation groups A-Q) have been identified in patients exhibiting
43 features consistent with FA (reviewed in Walden and Deans, 2014).

44 Whilst historically the FA/HR pathway has been associated with the HR-dependent repair
45 of ICLs, it plays a broader role in protecting cells from replication stress. Indeed, HR-deficient (and
46 some FA) cell lines are hypersensitive to replication stress-inducing agents that do not induce ICLs
47 (e.g. aphidicolin [APH] and hydroxyurea [HU]) (Howlett *et al.*, 2005). It has been proposed that FA
48 and HR proteins function to: (i) protect stalled/collapsed forks from uncontrolled nucleolytic attack
49 (which may render such forks unrecoverable and/or prone to inappropriate repair) (Schlachter *et al.*,
50 2011; 2012); and (ii) in some cases may facilitate their restart once repair is complete. Cells
51 defective in these processes exhibit an increase in under-replicated DNA, particularly at common
52 fragile sites (CFS). This can result in the generation of ultra-fine anaphase bridges (UFBs), and
53 can ultimately manifest as chromosome breakage and micronuclei (Naim and Roselli, 2009a). The
54 accumulation of such genetic damage over time eventually triggers cell death, and may contribute
55 to the attrition of highly replicating cells, such as germ and haematopoietic cells (Garaycochea
56 and Patel, 2014).

57 Despite extensive research, it is still not completely understood how the cell regulates
58 repair of replication damage via the FA/HR pathway. A wide variety of DNA damage response
59 (DDR) and DNA repair proteins, including components of the FA/HR pathway, are recruited to
60 stalled forks upon replication stress (Sirbu *et al.*, 2013). However, it is unclear how replication forks
61 requiring repair are marked: this might involve RPA coated ssDNA, specific DNA secondary
62 structures or damage-inducible post-translational modifications of the replication machinery and/or

63 surrounding chromatin. It is also likely that some repair proteins are constitutive components of the
64 replication fork machinery, to allow immediate initiation of the DDR once a lesion is encountered.

65 We have identified an uncharacterised factor, BOD1L, associated with newly replicated
66 chromatin. Cells lacking BOD1L accumulate catastrophic levels of genome damage following
67 replication stress, particularly after MMC exposure, manifesting as excessive chromosome
68 breakage. Although related to the mitotic regulator BOD1, we demonstrate that BOD1L does not
69 regulate spindle orientation but rather functions to protect stalled/damaged replication forks from
70 uncontrolled DNA2-dependent resection. We further show that BOD1L functions within the FA
71 pathway as part of the fork protection machinery, to stabilize Rad51 on chromatin by suppressing
72 the anti-recombinogenic and pro-resection activities of Fbh1 and BLM. Taken together, our data
73 establish that BOD1L is a critical factor associated with the replication machinery that acts to
74 promote fork stability by counteracting negative regulators of HR.

75

76 RESULTS

77 BOD1L is an uncharacterised factor that maintains genome stability following replication 78 stress

79 Isolation of proteins on nascent DNA (iPOND) is a robust method for the detection of
80 proteins at sites of newly replicated DNA (Sirbu *et al.*, 2013). However, many proteins are sensitive
81 to the harsh conditions of iPOND. To overcome this issue, we first modified the original iPOND
82 protocol and subsequently utilised this new method coupled with mass spectrometry to identify
83 factors associated with nascent chromatin. As with other iPOND-based proteomic studies (Sirbu *et al.*,
84 2013), we identified numerous replication machinery components at ongoing forks, including
85 the MCM helicase, PCNA, the RFC complex, RPA and the replicative polymerases, pol δ and pol α
86 (**Figure S1A**). In addition to these proteins, we also identified Biorientation Defect 1-like (BOD1L);
87 a large, previously uncharacterised protein with N-terminal homology to the mitotic regulator BOD1
88 (**Figure 1A**) (Porter *et al.*, 2007). Consistent with these data, we confirmed the presence of BOD1L
89 in EdU precipitates by Western blotting (**Figure 1B**). To verify that BOD1L associated with
90 replication fork proteins, we performed proximity-ligation assays with antibodies against PCNA and
91 BOD1L. We readily detected nuclear PLA signals in undamaged EdU-positive cells, which were
92 strongly reduced in EdU-negative and BOD1L-depleted cells (**Figures 1C-D**). Moreover, we could
93 co-immunoprecipitate BOD1L, and murine GFP-tagged Bod1L, with Mcm2 and Mcm7 (**Figures 1E**
94 **and S1B**). Together, these data indicate that BOD1L is localised at/near replication forks.

95 In addition to its homology to BOD1, the amino acid sequence of BOD1L also contains
96 several *in vivo* ATM/ATR phosphorylation sites (Matsuoka *et al.* (2007)), suggesting that BOD1L
97 might play a role in the DDR. To investigate this, we depleted cells of BOD1L using siRNA and
98 analysed cellular sensitivity to a range of DNA damaging agents. Knockdown of BOD1L exquisitely
99 hypersensitised cells to agents that induce replicative stress, in particular MMC (**Figures 1F and**
100 **S1C**), and was significantly more severe than loss of FANCA. However, co-depletion of FANCA
101 and BOD1L revealed that these two factors were epistatic for MMC hypersensitivity, suggesting
102 that BOD1L may function within the FA pathway. Treatment of BOD1L-depleted cells with
103 replication stress-inducing agents also induced increased micronuclei formation, indicating a
104 critical role for BOD1L in maintaining genome stability upon replication damage (**Figure 1G**).

105 Importantly, these observations were recapitulated in several cell lines, and in two independent
106 DT40 BOD1L knockout clones (**Figures S1D-H**), demonstrating that the genome instability
107 observed is specifically due to loss of BOD1L, and is neither cell line nor organism specific.

108

109 **BOD1L and BOD1 are functionally distinct**

110 BOD1 is a mitotic factor that associates with metaphase chromosomes and is essential for
111 correct orientation of the mitotic spindle (Porter *et al*, 2007; Porter *et al*, 2013). Given the sequence
112 similarity of the N-terminus of BOD1L to BOD1, it was conceivable that the increased micronuclei
113 observed in BOD1L-depleted cells arose from mitotic abnormalities.

114 To first investigate whether BOD1L was functionally related to BOD1, we performed
115 phenotypic analyses of cells depleted of either BOD1 or BOD1L by siRNA. Whilst loss of BOD1L
116 resulted in elevated DDR signalling, specifically the phosphorylation of H2AX and RPA2, this
117 defect was not observed in BOD1-depleted cells (**Figure 2A**). Furthermore, loss of BOD1 neither
118 increased MMC-induced micronuclei, nor engendered a cellular hypersensitivity to MMC or HU
119 (**Figures 2B-D**). In addition, unlike BOD1 depletion, loss of BOD1L did not cause mitotic or spindle
120 alignment defects (**Figures 2E-F**). Finally, immunostaining analyses of MMC-treated cells revealed
121 that BOD1L depletion increases the proportion of micronuclei that are acentric (CENPA-negative)
122 and that contain DNA double strand breaks (DSBs) (53BP1-positive), suggesting that these
123 micronuclei originated from unrepaired DNA damage (**Figure 3A**). Together, these observations
124 demonstrate that BOD1 and BOD1L perform separate roles in cell cycle regulation and genome
125 maintenance respectively.

126

127 **BOD1L functions within the Fanconi Anaemia pathway**

128 Micronuclei are observed in the absence of several genome stability factors, although FA-
129 deficient are especially prone to ICL-induced micronucleation (Naim and Rosselli, 2009a). It has
130 been proposed that micronuclei arising in cells undergoing replication stress stem from a failure to
131 complete timely DNA replication. This results in the persistence of under-replicated DNA in cells as
132 they enter mitosis, which can manifest as UFBs, typically marked by PICH and flanked by
133 FANCD2 foci (Chan *et al.*, 2007; Chan *et al.*, 2009). These can lead to chromosome breakage and

134 packaging of the damaged DNA into 53BP1 bodies visible in the subsequent G1 phase (Lukas *et*
135 *al.*, 2011).

136 We next determined the prevalence of late replicating DNA, UFBs and G1-phase 53BP1
137 bodies in BOD1L deficient cells following MMC exposure. Loss of BOD1L dramatically increased
138 the percentage of mitotic cells positive for either FANCD2/PICH positive UFBs or for EdU foci
139 following MMC exposure (**Figures 3B-D**). Consistent with this, we observed a significant rise in the
140 number of 53BP1 G1 bodies in these cells, which was again more severe than depletion of FANCA
141 alone (**Figures 3E-F**). Collectively, these data suggest that the micronuclei observed in BOD1L-
142 deficient cells are due to a failure to correctly resolve replication stress.

143 FA cells exhibit chromosomal hypersensitivity to agents that induce ICLs, caused in part by
144 the presence of under-replicated DNA, and exhibit chromatid breakage at specific loci, namely
145 CFS (Barlow *et al.*, 2013; Durkin *et al.*, 2007; Schoder *et al.*, 2010). Strikingly, loss of BOD1L
146 resulted in catastrophic levels of chromosome breakage following MMC exposure, which was
147 markedly more severe than FANCA loss (**Figures 3G and S2A**). In agreement with previous data,
148 co-depletion of FANCA/BOD1L had no additional effect on genome instability. Furthermore, the
149 majority of BOD1L-deficient cells showed evidence of chromosome breakage at the CFS locus
150 FRA16D (**Figure 3H**). Importantly, genome stability in BOD1L-depleted cells was restored by the
151 stable expression of murine Bod1L, which is resistant to siRNA-mediated degradation, in two
152 independent HeLa cell clones (CFlap-mBod1L C1-4 and C5-20) (**Figures S2B-D**). Taken together,
153 these observations clearly demonstrate that BOD1L functions to resolve replication stress, in
154 conjunction with FA pathway components (Naim and Rosselli, 2009b).

155 Given that BOD1L is epistatic with a core FA pathway component, we evaluated the
156 functional integrity of this pathway in the absence of BOD1L. The DNA damage-induced mono-
157 ubiquitylation of FANCD2 is a central event within the FA pathway, and is often used as a marker
158 of FA pathway integrity (Smogorzewska *et al.*, 2007). Loss of BOD1L had no effect on the focal
159 recruitment of FANCD2 to sites of MMC damage, nor its ability to be mono-ubiquitylated (**Figures**
160 **S2E-F**), suggesting that BOD1L functions downstream of the FA core and FANCI/D2 complexes
161 within the FA/HR pathway.

162

163 **BOD1L depletion compromises the fidelity of DNA replication following replication stress**

164 The cellular response to replication stress is primarily coordinated by the ATR kinase,
165 which activates the intra-S phase checkpoint and protects stalled replication forks from collapse.
166 Recent studies on ATR-deficient cells have demonstrated that fork protection is crucial to maintain
167 CFS integrity (Koundrioukoff *et al.*, 2013). Given that BOD1L-depleted cells exhibit CFS instability,
168 this suggests that BOD1L may protect replication forks directly, in a similar fashion to ATR. We
169 therefore hypothesised that loss of BOD1L would negatively impact S-phase regulation and/or
170 replication fork dynamics upon replication stress.

171 Initially, we sought to determine the effect of depleting BOD1L on cell cycle progression.
172 Compromising BOD1L expression had little effect on cell cycle progression in the absence of DNA
173 damage (**Figure 4A**). However, consistent with a defect in resolving S-phase damage, cells
174 lacking BOD1L rapidly accumulated in G2-phase following MMC exposure, with a concomitant
175 reduction in mitotic index (**Figures 4B and S3A-B**); a phenotype reminiscent of FA cells (Akkari *et al.*, 2001; Heinrich *et al.*, 1998;). Next, to directly analyse the stability of on-going replication forks
176 following HU treatment, we quantified the symmetry of sister replication forks originating from the
177 same origin and travelling in opposite directions. Since sister forks typically display similar
178 replication rates (Conti *et al.*, 2007), marked fork asymmetry indicates that individual forks are
179 more prone to stalling (Rodriguez-Lopez *et al.*, 2002). Supportive of a role for BOD1L in promoting
180 fork stability, we observed a significant increase in replication fork asymmetry in BOD1L depleted
181 cells following HU treatment (**Figure 4C**), suggesting that damaged forks are slower to restart
182 and/or are more susceptible to stalling in its absence.

184 We also observed a significant increase in new origin firing in response to both HU and
185 MMC in cells lacking BOD1L, which was dependent on Cdk1/2 activity (**Figures 4D-E and S3C-E**).
186 However, this origin firing was not due to defective ATR/Chk1 signalling, since BOD1L-depleted
187 cells were proficient for Chk1 phosphorylation on both S317 and S345 in response to MMC
188 (**Figure 4F**). This phenotype was also not present in cells depleted of BOD1, further strengthening
189 the functional divergence of these proteins (**Figure S3F**).

190 Despite observing increased new origin firing and replication fork asymmetry in BOD1L
191 depleted cells after replication stress, there was no concomitant reduction in the number of

192 restarted forks, nor any increase in fork stalling (first label terminations). One possible explanation
193 was that new origins were firing proximal to stalled/collapsed (red only labelled) forks in BOD1L
194 depleted cells, therefore artificially enhancing the quantification of restarted (red and green
195 labelled) replication forks. To investigate this, we used a Cdk1/2 inhibitor to inhibit new origin firing
196 in cells lacking BOD1L, and examined the impact on fork stalling/restart. Accordingly, we found
197 that inhibition of Cdk1/2 activity during HU exposure ablated new origin firing, increased the
198 prevalence of stalled replication forks, and decreased fork restart (**Figure S3G**), suggesting that
199 BOD1L prevents fork stalling upon replication stress.

200 Together, these data suggest that, upon the induction of replication stress, a lack of BOD1L
201 compromises fork stability and/or restart, which triggers dormant origin firing proximal to the
202 stalled/damaged fork.

203

204 **BOD1L protects stalled forks from uncontrolled resection**

205 It is thought that uncontrolled origin firing in the absence of ATR leads to excessive ssDNA
206 generation and exhaustion of cellular pools of RPA, which both cause irreversible fork collapse
207 (Toledo *et al.*, 2013). It is conceivable that global depletion of soluble RPA may also underlie the
208 excessive chromosome breakage observed in BOD1L deficient cells, since they also exhibit
209 increased origin firing, fork instability and defective fork restart. To investigate this, we first sought
210 to determine levels of ssDNA present in BOD1L-depleted cells following MMC exposure. Loss of
211 BOD1L resulted in a significant increase in RPA loading onto damaged chromatin compared to
212 control cells (**Figures 5A-B and S4A**). Moreover, ablation of BOD1L expression significantly
213 enhanced the formation of MMC-induced native BrdU foci (**Figures 5C and S4B**), consistent with
214 increased ssDNA generation in these cells. However, in contrast to ATR-deficient cells (Toledo *et al.*, 2013), RPA over-expression had no effect on either new origin firing or chromosomal instability
215 in the absence of BOD1L (**Figures S4C-E**). In addition, it is clear that the ATR-Chk1 pathway is
216 functional in BOD1L deficient cells (see **Figure 4F**). Therefore, although cells lacking ATR or
217 BOD1L display phenotypic similarities, it appears that the mechanisms underlying replication fork
218 stalling/collapse differ.

220 It has been demonstrated that loss of FA pathway components such as BRCA2 and
221 FANCD2 leads to nucleolytic degradation of stalled replication forks, rendering them non-
222 permissive for repair by HR (Schlacher *et al.*, 2011; 2012). This excessive fork resection underlies
223 the increased chromosome breakage exhibited by BRCA2-null cells. The nucleases Mre11 and/or
224 DNA2 appear to perform this uncontrolled resection; inhibition of Mre11 can alleviate fork
225 degradation in BRCA2 deficient cells (Schlacher *et al.*, 2011), and depletion of DNA2 rescues the
226 hypersensitivity of FANCD2 deficient cells to cisplatin (Karanja *et al.*, 2014). To investigate whether
227 similar mechanisms underlie the phenotypes of BOD1L-deficient cells, we examined levels of
228 MMC-induced RPA2 phosphorylation on S4/S8, a well-established marker of DNA resection.
229 Depletion of BOD1L (but not BOD1) resulted in elevated levels of RPA2-S4/8 phosphorylation
230 following MMC treatment (**Figures 5D-F and 2A**), which could be restored to control levels by the
231 expression of CFlap-mBod1L (**Figure S4F**). Hyper-phosphorylation of RPA2 was also observed in
232 BOD1L-deficient cells following exposure to HU, indicating that this defect is not restricted to MMC-
233 induced ICLs (**Figure S4G**). Therefore, these data suggest that BOD1L functions to suppress
234 resection. Consistent with this, we observed an increased localisation of BOD1L to damaged forks
235 undergoing resection (**Figure 5G**).

236 To confirm that uncontrolled resection in BOD1L depleted cells occurs specifically at
237 replication forks, we used the approach described by Schlacher *et al.* (2011) to monitor
238 degradation of nascent DNA. In line with previous observations, loss of BRCA1 or BRCA2
239 increased the degradation of newly synthesized DNA at forks (apparent as a decreased IdU:CldU
240 ratio; **Figure 6A and S5A-B**). Interestingly, cells lacking BOD1L exhibited similar degradation of
241 stalled replication forks. Critically, this was epistatic with either BRCA1 or BRCA2 depletion,
242 suggesting that BOD1L and BRCA1/2 function within the same pathway to protect replication forks.
243 In support, BRCA2 and BOD1L co-depletion had no additional effect on cellular hypersensitivity to
244 MMC or RPA2 hyper-phosphorylation compared to loss of the individual genes alone (**Figure S5C-
245 E**). Finally, BOD1L co-immunoprecipitated with the fork protection factors FANCD2 and BRCA2
246 (**Figure 6B**). Together, this provides strong evidence that BOD1L plays a vital role in preventing
247 unconstrained resection at stalled forks, in concert with FANCD2 and BRCA1/2.

248 We next sought to ascertain whether the increased resection seen in cells devoid of
249 BOD1L was mediated by Mre11, DNA2 and/or Exo1; three nucleases implicated in fork resection.
250 In line with the study by Karanja *et al.* (2014), co-depletion of DNA2, but not Exo1, completely
251 repressed MMC-induced RPA2 S4/8 hyper-phosphorylation observed in cells lacking BOD1L
252 (**Figures S5F-G**). Furthermore, the over-resection of stalled forks was completely abolished by co-
253 depletion of DNA2 with BOD1L. However, in contrast to previous findings with BRCA1/2 and
254 FANCD2 (Schlacher *et al.*, 2011), inhibition of Mre11 by Mirin was unable to rescue nucleolytic fork
255 degradation in the absence of BOD1L (**Figures 6C and S5H**). Moreover, the combined loss of
256 DNA2 and BOD1L restored the MMC-induced micronuclei and chromosome damage to normal
257 levels (**Figure 6D-E**). This demonstrates that the severe genome instability in cells lacking BOD1L
258 arises from uncontrolled DNA2-dependent resection of damaged forks.

259

260 **BOD1L stabilises Rad51 at damaged replication forks by suppressing anti-recombinogenic** 261 **pathways**

262 The strand exchange protein Rad51 is best known as a principal effector of HR, but it also
263 plays a central role in stabilising/promoting the restart of damaged replication forks (Petermann *et al.*,
264 2010; Costanzo, 2011). Accordingly, the excessive fork degradation observed in BRCA2 or
265 FANCD2 deficient cells is restored by overexpressing an ATPase-dead Rad51 mutant, which
266 stabilises Rad51 nucleofilaments on ssDNA by preventing its ATP-dependent dissociation
267 (Schlacher *et al.*, 2011; 2012). Thus, the loading of Rad51 onto stalled forks prevents uncontrolled
268 nucleolytic activity.

269 To investigate whether a defect with Rad51 function underlies the excessive fork resection
270 observed in the absence of BOD1L, we exposed BOD1L-depleted cells to MMC, and then
271 monitored the accumulation/retention of Rad51 at sites of damage by immunofluorescence.
272 Notably, MMC-induced Rad51 foci formation was severely compromised in BOD1L-depleted cells
273 (**Figure 6F**). This was observed with 4 different BOD1L siRNA sequences, and was not due to any
274 alteration in Rad51 protein expression (**Figures S5I-J**). Moreover, the defective focal recruitment
275 of Rad51 (**Figure S6A**) upon damage could be restored by the expression of CFlap-mBod1L
276 (**Figures S6B-C**). In keeping, Rad51 also failed to load efficiently onto MMC-damaged chromatin

277 in cells lacking BOD1L (**Figure 6G**). Consistent with this, BOD1L-depleted cells exhibited
278 increased numbers of MMC-induced radial chromosomes, and a concomitant decrease in the
279 frequency of MMC-induced SCEs (**Figures S6D-E**). Strikingly, the formation of IR-induced Rad51
280 foci at DSBs was unaffected in BOD1L-depleted cells (**Figure S6F**), suggesting that our
281 observations were not due to a global defect in Rad51 loading *per se*, but rather an inability to
282 localise/stabilise Rad51 to stalled replication forks.

283 Conceivably, a defect in recruiting Rad51 to stalled forks may be due to either a failure to
284 properly load Rad51 onto ssDNA, or an inability to maintain loaded Rad51 on chromatin. To
285 investigate the former possibility, we examined the impact of BOD1L depletion on the recruitment
286 of BRCA1, BRCA2 and PALB2, which are essential for Rad51 loading to ICLs (Bhattacharyya *et al.*,
287 2000; Godthelp *et al.*, 2006; Xia *et al.*, 2007). Cells lacking BOD1L exhibited no observable
288 defects in the re-localisation of BRCA1, BRCA2 or GFP-PALB2 to foci following exposure to MMC
289 (**Figures 6H and S6G-I**), suggesting that BOD1L may instead be required to stabilise/retain Rad51
290 on damaged chromatin.

291 Proteins involved in Rad51 filament dissolution play a vital role in controlling HR and
292 maintaining genome stability. Of these, the RecQ-like helicases BLM and RECQL5, and the F-box-
293 containing helicase Fbh1 are the best studied: all three suppress Rad51-dependent HR,
294 particularly in response to replication stress. We speculated that the phenotypes observed in
295 BOD1L-deficient cells following MMC exposure may arise from the uncontrolled activity of one or
296 more of these anti-recombinase(s). We therefore siRNA-depleted BOD1L in combination with
297 BLM, Fbh1 or RECQL5, and monitored the levels of MMC-induced RPA S4/S8 phosphorylation
298 and Rad51 foci formation. Strikingly, loss of either Fbh1 or BLM (but not RECQL5) reduced MMC-
299 induced RPA S4/S8 phosphorylation and restored Rad51 focus formation in BOD1L-depleted cells
300 (**Figures 7A-B and S7A-C**). Consistent with this, co-depletion of Fbh1 in BOD1L-depleted cells
301 restored MMC-induced loading of Rad51 onto chromatin (**Figure S7D**). However, depletion of
302 Fbh1 or BLM was unable to restore Rad51 focus formation in the absence of BRCA2, suggesting
303 that BOD1L acts downstream of BRCA2 to control Rad51 (**Figure S7E**). Depleting Fbh1 or BLM
304 expression also partially alleviated the over-resection of stalled replication forks observed in cells
305 lacking BOD1L, in keeping with the notion that Rad51 suppresses aberrant fork resection.

306 Strikingly, RECQL5 depletion further increased fork resection in the absence of BOD1L, indicating
307 that these two factors act in separate pathways (**Figures 7C and S7F**). Lastly, ablating Fbh1 or
308 BLM expression also restored genome stability in cells depleted of BOD1L (**Figures 7D and S7G**).
309 Loss of RECQL5, however, had no restorative impact on MMC-induced chromosomal instability.
310 Finally, and in keeping with a role for BOD1L in stabilising Rad51 by counteracting BLM, both BLM
311 and Rad51 could be co-immunoprecipitated with BOD1L or CFlap-mBod1L (**Figure 7E and S7H**).
312 Taken together, these data demonstrate that BOD1L functions to restrain the pro-resection
313 and anti-recombinogenic functions of BLM/Fbh1 towards Rad51, thereby stabilising Rad51 on
314 chromatin and promoting HR-dependent repair of damaged replication forks. In the absence of
315 BOD1L, damaged replication forks undergo deleterious DNA2-dependent nucleolytic resection,
316 which compromises fork repair/restart and leads to catastrophic genome instability (**Figure 7F**).

317 **DISCUSSION**

318 The ability to efficiently resolve replication stress is vital to maintain genome stability. In this
319 study, we have identified BOD1L as a factor associated with newly replicated chromatin that
320 functions to prevent catastrophic DNA damage induced by replication stress by protecting
321 damaged replication forks from promiscuous nucleolytic degradation.

322

323 **Loss of Rad51-mediated fork protection underlies uncontrolled fork resection and genome** 324 **instability in BOD1L deficient cells**

325 Rad51-dependent HR plays an essential role to stabilise, protect and promote the restart of
326 stalled or damaged replication forks. Central to this process is the BRCA1/BRCA2/PALB2-
327 dependent loading of Rad51 onto RPA-coated ssDNA generated at such forks (Costanzo, 2011).
328 Rad51 fork loading stabilises replication fork intermediates and prevents deleterious nucleolytic
329 processing (Petermann *et al.*, 2010; Schlacher *et al.*, 2011; 2012). Loss of this protective activity
330 cripples the repair/restart of damaged forks and compromises genomic integrity.

331 We observed that BOD1L depleted cells exhibit increased fork degradation in a manner
332 comparable to BRCA1/BRCA2 deficient cells. This suggests that defects in the recruitment and/or
333 stabilisation of Rad51 allow degradation of damaged forks in BOD1L-deficient cells. Yet, in marked
334 contrast to the complete loss of BRCA2 or PALB2 (Yuan *et al.*, 1999; Xia *et al.*, 2006; Zhang *et al.*,
335 2009), loss of BOD1L does not impact on the recruitment of Rad51 to DSBs induced by IR. This
336 implies that the function of BOD1L in regulating Rad51 in response to genotoxic damage is
337 restricted to lesions that cause replication stress.

338 The repair and restart of forks requires the tightly regulated processing of replication
339 damage (such as ICLs) by several different nucleases. Whilst such nucleolytic processing is
340 important for cell survival, uncontrolled activity of these nucleases is also detrimental to genomic
341 integrity (Adamo *et al.*, 2010; Karanja *et al.*, 2014). In keeping with this, uncontrolled resection of
342 damaged forks and increased genomic instability observed in the absence of BOD1L was
343 completely alleviated by co-depletion of DNA2. In contrast, inhibition of Mre11 activity with Mirin
344 had no effect on the degradation of stalled replication forks in the absence of BOD1L. Since both
345 BRCA2 and FANCD2 suppress the activity of Mre11 at stalled forks (Schlacher *et al.*, 2011; 2012;

346 Ying *et al.*, 2012), our data suggests that BOD1L acts independently to inhibit aberrant DNA2
347 activity. Based on this, loss of BOD1L and BRCA2 should further increase fork resection, rather
348 than exhibit the epistatic relationship we observed. Although the underlying reason is unclear, we
349 postulate that nucleolytic degradation of a damaged fork by one nuclease prohibits further
350 processing by other nucleases.

351 Interestingly, since BOD1L co-immunoprecipitated with both BRCA2 and FANCD2 in
352 unperturbed cells, this raises two intriguing possibilities: that multiple fork protection factors act to
353 individually block the activities of different nucleases towards replication forks, and that they may
354 exist in a single complex.

355

356 **Mechanisms for BOD1L in stabilising Rad51 on damaged chromatin**

357 Our data demonstrates that loss of BOD1L is epistatic with deficiencies in BRCA1/BRCA2,
358 although the phenotypes observed in the absence of BOD1L cannot be explained by an inability to
359 recruit BRCA1, BRCA2 or PALB2 to sites of replication stress. Instead, co-depletion of BOD1L with
360 the anti-recombinogenic helicases Fbh1 or BLM restored Rad51 focal recruitment, stalled
361 replication fork resection and genome stability. Thus, chromatin-bound Rad51 may be more
362 susceptible to anti-recombinases, and/or Rad51 nucleofilaments may be more unstable, in the
363 absence of BOD1L. Importantly, Fbh1 or BLM knockdown failed to recover MMC-induced Rad51
364 foci formation in BRCA2-depleted cells, suggesting that BOD1L acts downstream of
365 BRCA2/PALB2 (see **Figure S6F**). Moreover, BOD1L associates with Rad51, suggesting that it
366 may stabilise Rad51 directly. Together, our data suggest that BRCA2 and BOD1L function
367 independently in a common pathway to protect replication forks, and that BOD1L acts in a similar
368 manner to the C-terminus of BRCA2 (Esashi *et al.*, 2007; Schlacher *et al.*, 2011), i.e. promoting
369 Rad51 nucleofilament stability. Intriguingly, co-depletion of BOD1L with another anti-
370 recombinogenic helicase, RECQL5, failed to restore Rad51 foci formation, and actually
371 increased/accelerated fork degradation. This is in line with recent data demonstrating that the
372 combined loss of FA proteins with RECQL5 is additive in terms of fork degradation, and that BLM
373 and RECQL5 have divergent functions in the absence of an intact FA pathway (Kim *et al.*, 2015).

374 It is unclear why depletion of two independent factors (namely BLM or Fbh1) is able to
375 compensate for a lack of BOD1L, although BLM and Fbh1 have partially redundant functions in
376 DT40 cells (Kohzaki *et al.*, 2007). Whilst Fbh1 and BLM both have pro- and anti-recombinogenic
377 activities (Bugreev *et al.*, 2007; Fugger *et al.*, 2009), BLM can displace Rad51 from ssDNA, and
378 can also potentiate HR through its ability to stimulate DNA2-dependent end-resection by binding to
379 RPA (Chen *et al.*, 2013; Xue *et al.*, 2013; Sturzenegger *et al.*, 2014). It is possible that loss of
380 BLM activity in BOD1L/BLM knockdown cells has two effects: (1) increases Rad51 filament
381 stability and; (2) compromises DNA2-dependent resection of damaged forks, the latter of which
382 causes the genome instability apparent in BOD1L deficient cells.

383 Currently it is unknown whether BOD1L influences the activity of these anti-recombinases
384 directly or controls their access to the damaged replication fork and/or the Rad51 filament itself.
385 Given that BOD1L and BLM co-associate, it is tempting to speculate that BOD1L regulates BLM
386 activity directly. Alternatively, since the Rad51 paralogues stabilise Rad51 nucleofilaments by
387 blocking the translocase activities of anti-recombinogenic helicases (Amunugama *et al.*, 2013; Liu
388 *et al.*, 2011), BOD1L may act in an analogous fashion to regulate access of BLM/Fbh1 to Rad51,
389 ultimately stabilising Rad51 nucleofilaments at damaged replication forks. As a consequence,
390 ablating BOD1L could promote uncontrolled BLM-DNA2-dependent resection, and allow
391 BLM/Fbh1-dependent dissolution of Rad51 filaments.

392

393 **BOD1L functions in the latter stages of the FA/HR pathway**

394 The phenotypic similarities between BOD1L-deficient cells and FA-defective cells,
395 particularly after ICL induction, suggest that BOD1L functions as part of the FA/HR pathway.
396 Indeed, loss of BOD1L and core/downstream FA components (namely FANCA and BRCA2) are
397 epistatic for MMC hypersensitivity and fork protection. In further support, the increased fork
398 resection apparent in both BOD1L-deficient and FANCD2-null cells is attributable to the nucleolytic
399 activity of DNA2 (Karanja *et al.*, 2014). However, since BOD1L is not required for mono-
400 ubiquitylation or relocalisation of FANCD2 to sites of DNA damage, and also that the chromatin
401 localisation/retention of Rad51 is unaffected in cells lacking FA core components or FANCD2/I
402 (Ohashi *et al.*, 2005; Godthelp *et al.*, 2006), this indicates that BOD1L functions in the latter stages

403 of the FA pathway, downstream of FANCD2/I. This also suggests that fork protection mechanisms
404 independent of Rad51-loading (but perhaps dependent on Rad51 activity) also exist.

405

406 **Increased origin firing contributes to genome instability in BOD1L deficient cells**

407 We have shown that cells depleted of BOD1L exhibit increased new origin firing following
408 the induction of replication stress. We hypothesise that this elevated origin firing is a cellular
409 response to an inability to complete DNA replication, caused by the uncontrolled resection of
410 stalled forks due to a failure to stabilise Rad51. This results in elevated levels of mitotic replication,
411 UFBs, G1 53BP1 bodies and severe chromosomal instability.

412 Whether Rad51 defects alone promote new origin firing is currently unclear. Increased new
413 origin firing does not occur in human cells depleted of Rad51 or in BRCA2-null CHO cells following
414 HU (Petermann *et al.*, 2010; Jones *et al.*, 2014), but increased new origin firing has been shown in
415 BLM-deficient cells and those lacking PALB2 (Davies *et al.*, 2007; Nikkila *et al.*, 2013). It therefore
416 remains to be determined whether the inability of BOD1L-depleted cells to retain Rad51 at stalled
417 replication forks contributes to the increase in origin firing. In spite of this, we predict that the
418 increased origin firing in BOD1L deficient cells could contribute to genome instability, perhaps due
419 to collisions between newly fired origins and damaged forks lying in close proximity.

420

421 **Summary**

422 Taken together, our data leads us to propose the following model (**Figure 7F**): BOD1L
423 forms an essential component of the fork protection machinery. Upon stalling of a replication fork
424 (for example by an ICL), limited nucleolytic resection allows Rad51-dependent HR and
425 repair/restart of the stalled fork. BOD1L acts to stabilise Rad51 at such structures by protecting
426 Rad51 nucleofilaments from the activities of Fbh1/BLM. In the absence of BOD1L, Rad51 is
427 displaced from ssDNA by Fbh1/BLM, rendering the fork susceptible to uncontrolled resection by
428 DNA2, and leading to catastrophic genome instability, in part mediated by the presence of under-
429 replicated DNA.

430

431 **EXPERIMENTAL PROCEDURES**

432

433 **Cell culture and generation of cell lines**

434 A549, HeLa, HeLa S3, H1299 and HeLa-FUCCI cells were grown in Dulbecco's modified Eagle's
435 medium supplemented with 10% fetal bovine serum (FBS) (Gibco) and penicillin/streptomycin.
436 HeLa-CFlap-BOD1L and U-2-OS SUPER-RPA cells were cultivated as above in the presence of
437 200 µg/ml Geneticin. U-2-OS and U-2-OS-PALB2-GFP cells were cultured in McCoy's 5A medium,
438 supplemented with 10% FBS and penicillin/streptomycin. DT40 cells were cultured in RPMI 1640
439 medium (Invitrogen) supplemented with 7% FBS, 3% chicken serum, and 10 µM β-
440 mercaptoethanol. Further details of DT40s, HeLa-CFlap-mBOD1L cells, siRNA transfections and
441 clonogenic survival assays are given in the Extended Experimental Procedures.

442

443 **iPOND**

444 iPOND was performed on HeLa S3 as described previously (Sirbu *et al.*, 2013) with some
445 modifications to allow for improved detection of high molecular weight proteins, which are
446 described in Extended Experimental Procedures. In brief, newly synthesized DNA was labelled
447 with 10µM EdU, cells were fixed in 1% formaldehyde, permeabilised and the Click reaction was
448 performed using Azide-PEG (3+3)-S-S-Biotin Conjugate (Click ChemistryTools). Following
449 sonication, EdU labelled DNA was precipitated using Streptavidin beads and eluted in buffer
450 containing DDT.

451

452 **Statistical analyses**

453 Differences in survival assays were analysed by two-way ANOVA. Statistical differences in all
454 cases were determined by Student's t-test, except for fork asymmetry, which was analysed by
455 Mann-Whitney rank sum test. In all cases: NS = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

456 **AUTHOR CONTRIBUTIONS**

457 MRH, JJR and GSS designed the study, performed experiments and wrote the manuscript. AW
458 performed iPOND. VB made HeLa-CFlap-mBOD1L cells and performed CFlap IPs. ANB and JN
459 created BOD1L-null DT40s. ESM and AZ performed experiments and created reagents. ELR
460 assisted with DNA combing. ND performed FISH. TS, SJB and WN supervised and advised on
461 experiments. All authors contributed to manuscript revisions.

462

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473

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668

669 **FIGURE LEGENDS**

670 **Figure 1: BOD1L is present at newly-replicated DNA, and ensures cellular viability after**
671 **replication stress. (A)** *Upper:* Schematic of human BOD1L and BOD1 domain structure and
672 ATM/ATR phosphorylation sites. *Lower:* Amino acid sequence alignment of BOD1L and BOD1.
673 Conserved residues (red) and similar residues (+) are denoted. **(B)** Immunoblotting of EdU-
674 coprecipitates from HeLa S3 cells. **(C-D)** HeLa cells were transfected with the indicated siRNAs,
675 and pulsed with 10 μ M EdU for 10 minutes before pre-extraction/fixation. EdU incorporation was
676 visualised with Click-iT chemistry, and detection of protein-protein associations were performed
677 using a fluorescently labelled PLA probe along with the indicated antibodies. (C) shows
678 quantification of PLA signals/nucleus from at least 100 cells ($n = 3$; lines denote mean values), and
679 representative images are shown (D). Scale bars = 10 μ m. **(E)** HeLa nuclear cell extracts were
680 subjected to IP with the indicated antibodies, and inputs and immunoprecipitates were analyzed by
681 immunoblotting. Blots originate from a single gel. A white line denotes removal of irrelevant lanes.
682 **(F)** The survival of HeLa cells transfected with the indicated siRNA following exposure to
683 mitomycin C (MMC) or hydroxyurea (HU) was assessed by colony survival assay. **(G)** Micronuclei
684 formation following DNA damage was assessed in siRNA-transfected HeLa cells by fluorescence
685 microscopy. Plots (F)-(G) represent mean data from four independent experiments; error bars =
686 SEM. See also Figure S1.

687

688 **Figure 2: BOD1L is functionally distinct from BOD1. (A).** Whole cell extracts (WCE) of HeLa
689 cells transfected with the indicated siRNA were analysed by immunoblotting after exposure to 50
690 ng/ml MMC for the denoted times. **(B)** HeLa cells from (A) were exposed to 50 ng/ml MMC for 24
691 h, and micronuclei enumerated. **(C-D)** The survival of HeLa cells transfected with the indicated
692 siRNA was assessed by colony survival assay as in **Figure 1F**. **(E-F)** Untreated HeLa cells from
693 (A) were immunostained with antibodies to α -tubulin and PCNT1, and the percentage of mitotic
694 cells in each stage of mitosis (E), or their ability to form centrosomes (F), was analysed by
695 immunofluorescence. Scale bars = 10 μ m. Data represent mean \pm SEM of three independent
696 experiments.

697

698 **Figure 3: BOD1L knockdown leads to problematic resolution of replication stress. (A)** The
699 percentage of micronuclei positive for either 53BP1 or CENPA was quantified by
700 immunofluorescence microscopy in HeLa cells transfected with indicated siRNAs after exposure to
701 50 ng/ml MMC for 24 h. **(B)** The percentage of mitotic cells with PICH-positive UFBs was
702 quantified in transfected HeLa cells after exposure to 250 ng/ml MMC for 3 h and release into fresh
703 media for 36 h. **(C)** The mean percentage of PICH-positive UFBs with terminal FANCD2 foci in
704 mitotic cells from (B) is indicated. **(D)** Cells from (B) were pulsed with 10 μ M EdU for 45 minutes
705 before fixation. Mitotic EdU incorporation was visualised with Click chemistry labelling, and the
706 mean number of EdU foci per mitotic cell, and merged representative images, are shown. Scale
707 bars = 10 μ m. **(E-F)** HeLa-FUCCI cells were siRNA-transfected, and exposed to 50 ng/ml MMC for
708 24 h. (E) WCE were analysed by immunoblotting. Loading control denotes a non-specific protein
709 detected by anti-BOD1L antibody. (F) The number of 53BP1 bodies in RFP-positive (i.e. G1) cells
710 was enumerated. **(G)** Damage to metaphase chromosomes from HeLa cells subjected to the
711 indicated siRNAs was determined by Geimsa staining and light microscopy. *Upper:* Graphs
712 integrate data from three independent experiments (n = 150; lines denote mean values). *Lower:*
713 Representative metaphase spreads are shown, with chromosomal damage denoted by
714 arrowheads. **(H)** Cells from (G) were analysed by FISH using probes against FRA16D. Plots
715 represent mean \pm SEM of three independent experiments. See also Figure S2.

716

717 **Figure 4: BOD1L knockdown increases origin firing after replication stress and destabilises**
718 **replication forks. (A-B)** The cell cycle profiles of HeLa cells subjected to the indicated siRNAs
719 were analysed by flow cytometry. Representative profiles from untreated cells (A) or after exposure
720 to 250 ng/ml MMC for 3 h (B) are shown. **(C-D)** DNA fibre analysis of HeLa cells transfected with
721 the indicated siRNAs. Cells were pulsed with CldU, exposed to 2 mM HU for 2 h, and pulsed with
722 IdU. Plots indicate ratios of left/right fork lengths of bidirectional replication forks travelling from a
723 single origin. Lines denote median ratios (C). DNA fibres were enumerated, and the percentage of
724 new origins (IdU-labelled only) is displayed (D). **(E)** Transfected cells from (C) were exposed to 50
725 ng/ml MMC for 24 h, and pulsed sequentially with CldU and IdU. DNA fibres were quantified, and
726 the percentage of new origins is displayed. **(F)** WCE of HeLa cells transfected as above and

727 exposed to MMC for the indicated times were analysed by immunoblotting. *Chronic = 50 ng/ml
728 MMC. **Acute = 250 ng/ml MMC for 3h followed by wash out. Times for acute exposure indicate h
729 post washout. Plots represent mean \pm SEM of at least three independent experiments. See also
730 Figure S3.

731

732 **Figure 5: BOD1L prevents excessive ssDNA formation and RPA2 hyper-phosphorylation**
733 **after MMC exposure. (A)** Soluble and chromatin fractions of U-2-OS cells transfected with the
734 indicated siRNAs, and exposed to 100 ng/ml MMC for 24 h, were analysed by immunoblotting.
735 Loading control denotes a non-specific protein detected by anti-BOD1L antibody. Blots originate
736 from a single gel. A white line denotes removal of superfluous lanes. **(B)** RPA foci formation was
737 analysed in HeLa cells transfected as above and exposed to 50 ng/ml MMC for the denoted times.
738 **(C)** Native BrdU foci formation in U-2-OS cells by fluorescence microscopy. Cells were transfected
739 with the indicated siRNAs, and BrdU added for 24 h. Cells were exposed to 50 ng/ml MMC for a
740 further 24 h in the presence of BrdU, and immunostained with antibodies to BrdU and γ H2AX. Foci
741 formation was analysed (see **Figure S4B**), and enumerated. **(D)** WCE of HeLa cells transfected as
742 in (B), and exposed to MMC for the indicated times, were analysed by immunoblotting. *Chronic =
743 50 ng/ml MMC. **Acute = 250 ng/ml MMC for 3h followed by wash out. Times for acute exposure
744 indicate h post washout. **(E)** Phospho-RPA (S4/S8) and RPA foci formation in transfected HeLa
745 cells exposed to 50 ng/ml MMC for 24 h. **(F)** The number of double positive cells from (E) was
746 enumerated. **(G)** Detection of protein-protein interactions was performed using a fluorescently
747 labelled PLA probe in HeLa cells from (B). The plot shows quantification of PLA signals/nucleus
748 from at least 100 cells (n=3; lines denote mean values), and representative images are shown.
749 Plots represent mean \pm SEM of three independent experiments. Scale bars = 10 μ m. See also
750 Figure S4.

751

752 **Figure 6: BOD1L is required to suppress aberrant fork resection after replication stress, and**
753 **is required for efficient Rad51 chromatin loading. (A)** Fork degradation was analysed in U-2-
754 OS cells. Cells were transfected with the indicated siRNAs, pulsed for 20 min each with CldU and

755 IdU, and exposed to 4 mM HU for 5 h. DNA was visualised with antibodies to CldU and IdU, and
756 plots denote the average ratios of IdU:CldU label lengths from three independent experiments.
757 Arrows indicate mean values (see **Figure S5B**). **(B)** HeLa nuclear cell extracts were subjected to
758 IP with the denoted antibodies, and inputs and immunoprecipitates were analyzed by
759 immunoblotting. Blots originate from a single gel. A white line denotes removal of irrelevant lanes.
760 **(C)** Fork degradation in U-2-OS cells transfected and treated as in (A) was analysed. Where
761 appropriate cells were treated with Mirin for the duration of the HU pulse (see **Figure S5H**). **(D)**
762 Micronuclei formation was quantified in HeLa cells transfected with the indicated siRNAs and
763 treated with 50 ng/ml MMC for 24 h. **(E)** Damage to metaphase chromosomes from cells (D) was
764 analysed (n = 150; lines denote mean values). **(F)** Rad51 foci formation was analysed in siRNA-
765 transfected HeLa cells, and exposed to 50 ng/ml MMC for the indicated times. Scale bars = 10 μ m.
766 **(G)** Soluble and chromatin fractions from **Figure 5A** were analysed by immunoblotting. **(H)** Foci
767 formation of BRCA1 and BRCA2 was analysed in HeLa cells from (F). Alternatively, U-2-OS-
768 PALB2-GFP cells were transfected with the indicated siRNAs, exposed to 50 ng/ml MMC for 24 h,
769 and fixed. In both cases mean percentage of cells with foci are shown (see **Figures S6G-I**). Plots
770 (D-H) represent mean \pm SEM of three independent experiments. See also Figures S5 and S6.

771

772 **Figure 7: BOD1L stabilises Rad51 chromatin loading to prevent excessive replication fork**
773 **resection. (A-B)** RPA2/phospho-RPA2 S4/S8 (A) or Rad51 (B) foci formation was analysed in
774 HeLa cells transfected with the indicated siRNAs and exposed to 50 ng/ml MMC for 24 h. Scale
775 bars = 10 μ m. **(C)** Fork degradation was assessed in U-2-OS cells transfected with the indicated
776 siRNAs as described in **Figure 6A** (see also **Figure S7F**). **(D)** Damage to metaphase
777 chromosomes in HeLa cells from (A) was analysed (n = 100; lines denote mean values). **(E)** HeLa
778 nuclear cell extracts subjected to IP with the indicated antibodies, and inputs and
779 immunoprecipitates were analyzed by immunoblotting. Blots originate from a single gel. A white
780 line denotes removal of irrelevant lanes. Plots (C-D) represent mean \pm SEM of three independent
781 experiments. **(F)** Model of BOD1L function to promote Rad51 nucleofilament stability and prevent
782 uncontrolled resection of replication forks. Upon replication fork stalling, forks undergo minimal
783 nucleolytic processing (i), allowing Rad51 loading/protection by BRCA1/BRCA2/PALB2 (ii). BOD1L

784 acts to stabilise Rad51 nucleofilaments by protecting them from the activities of BLM/Fbh1 (iii),
785 thus preventing uncontrolled resection and allowing Rad51-mediated repair/restart of forks,
786 ultimately maintaining genome stability. In the absence of BOD1L, BLM/Fbh1 act to remove Rad51
787 from such forks exposing them to uncontrolled DNA2-dependent processing (iv). To compensate
788 for this fork instability, increased new origin firing occurs. When combined with uncontrolled
789 resection of replication forks, this leads to catastrophic genome instability (v). See also Figure S7.

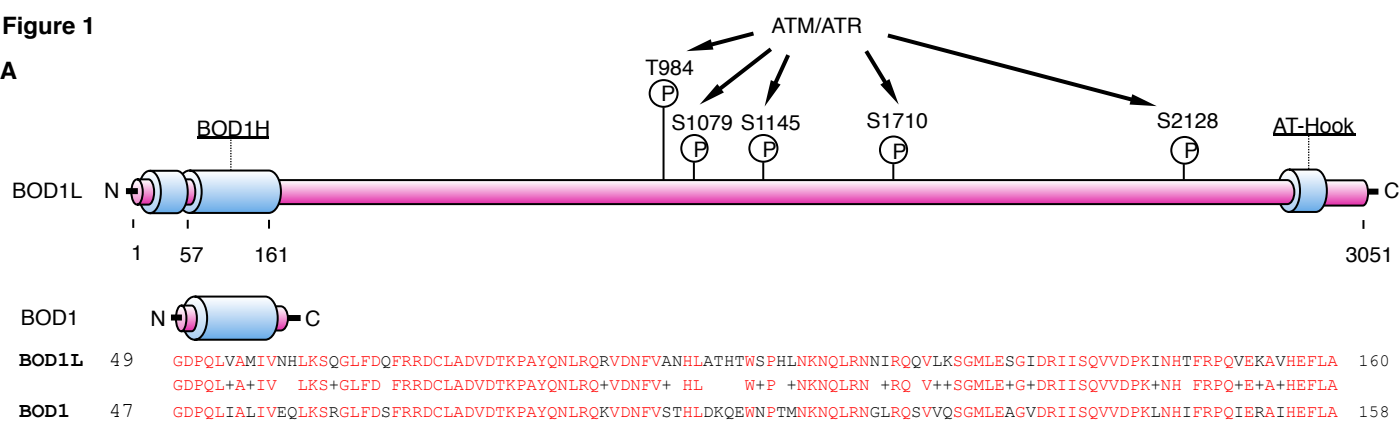
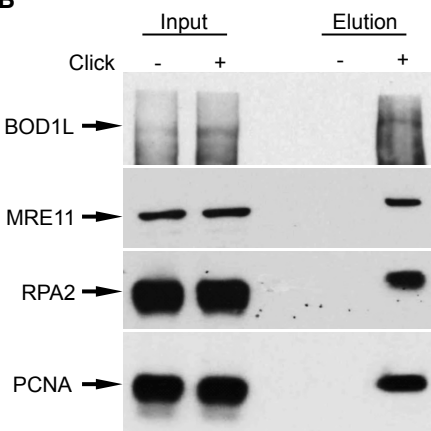
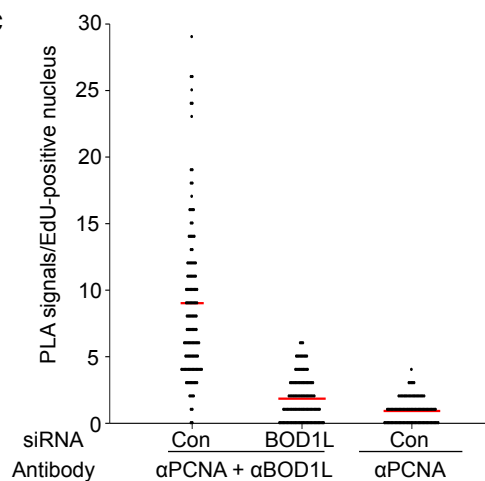
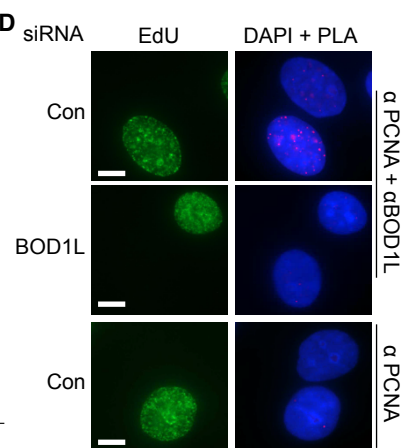
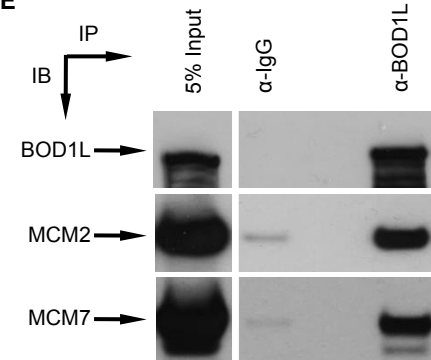
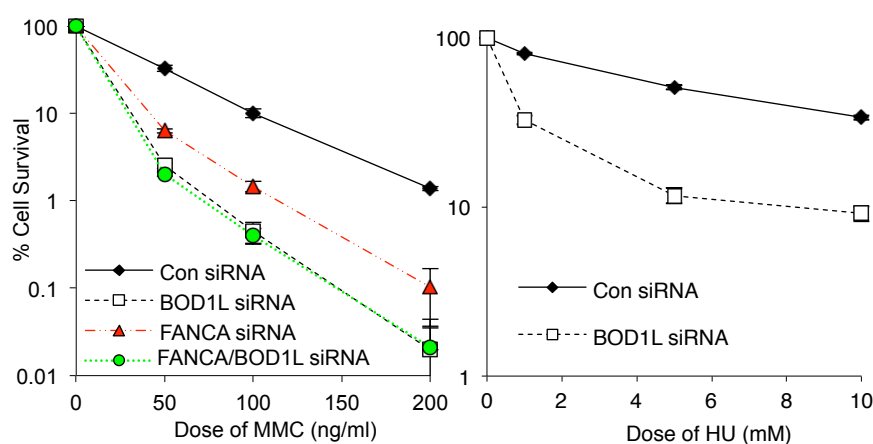
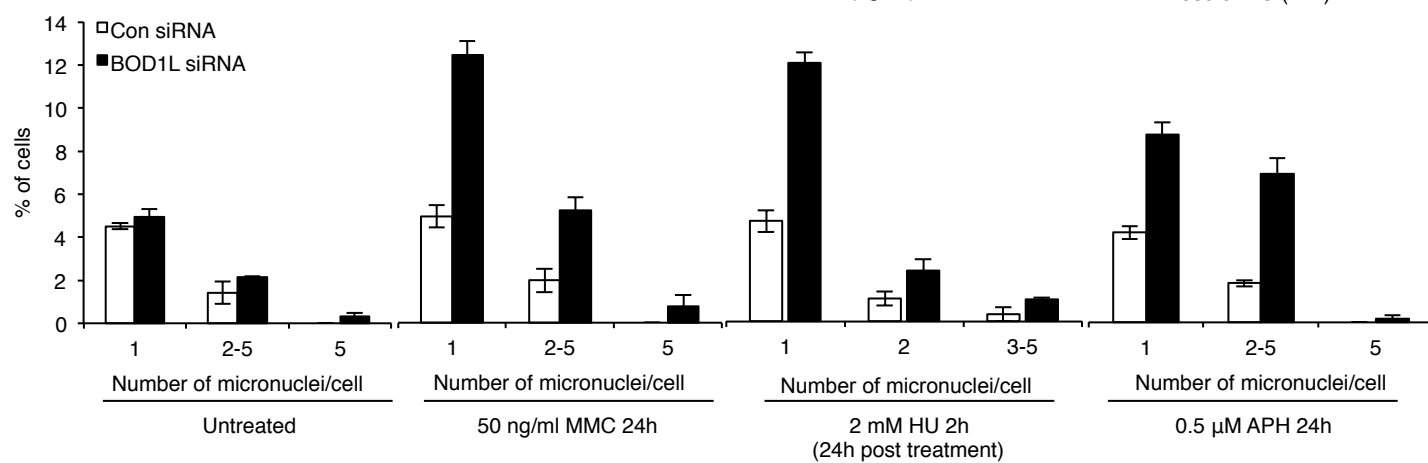
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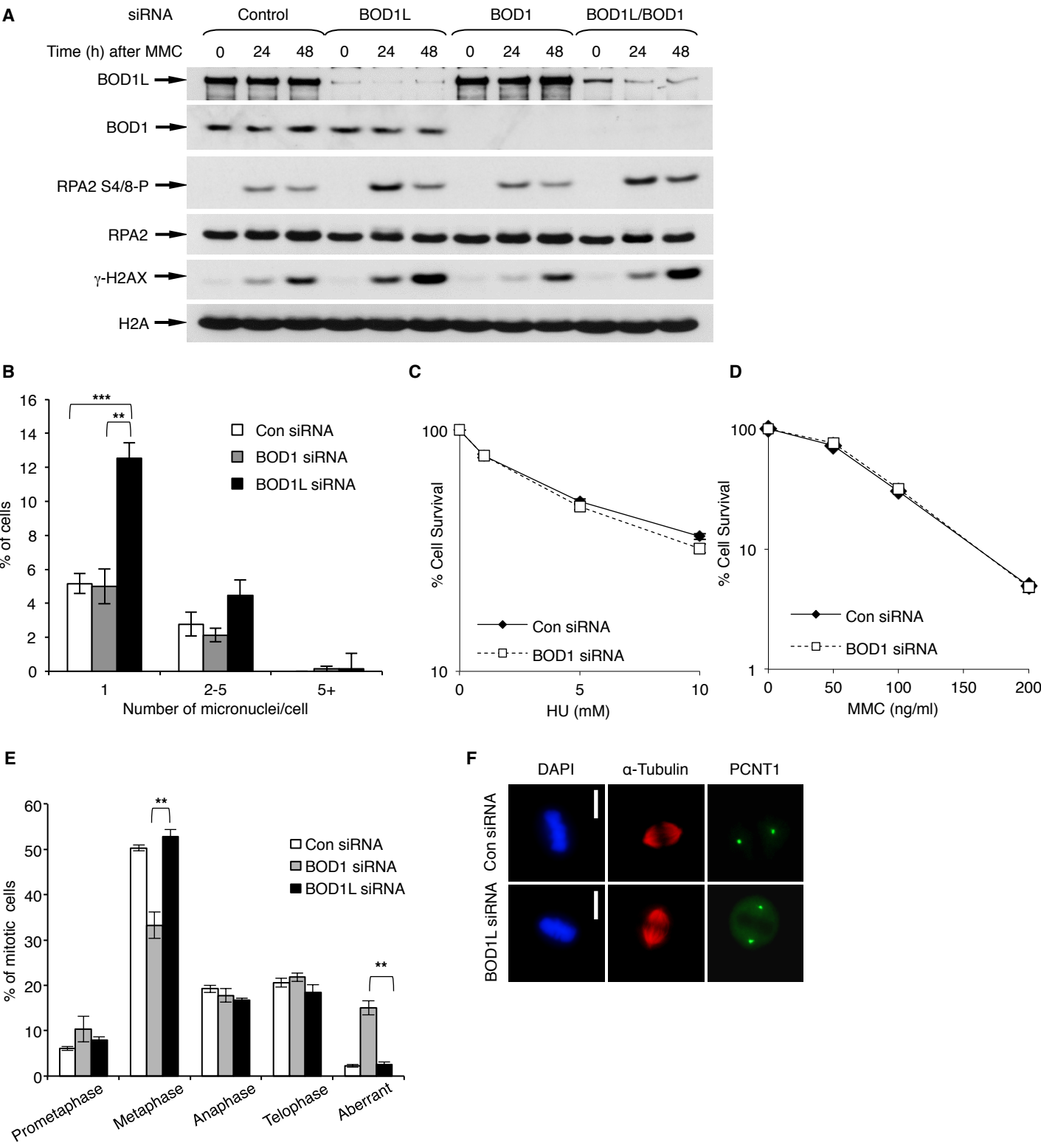


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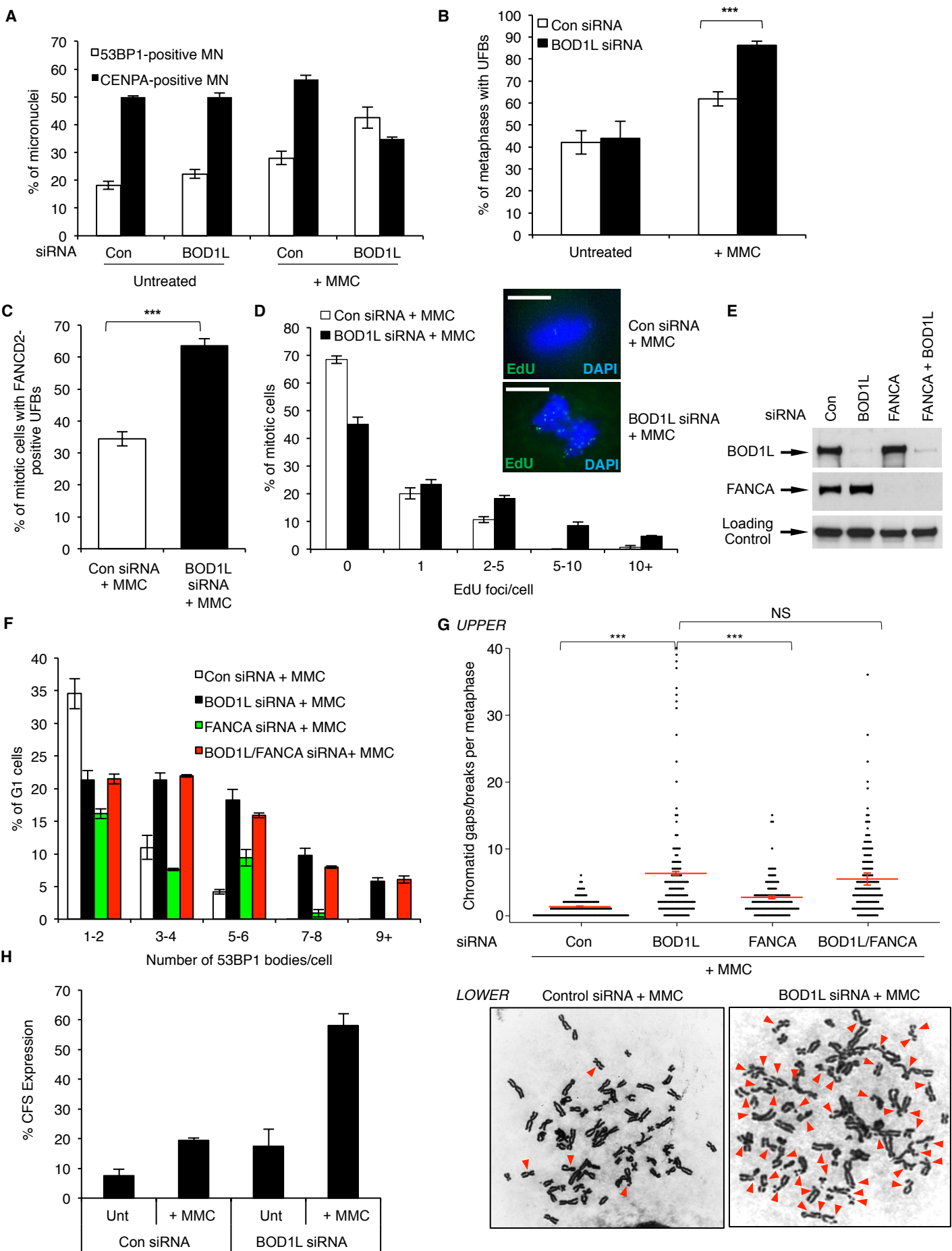
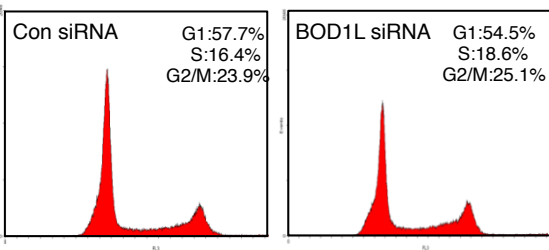
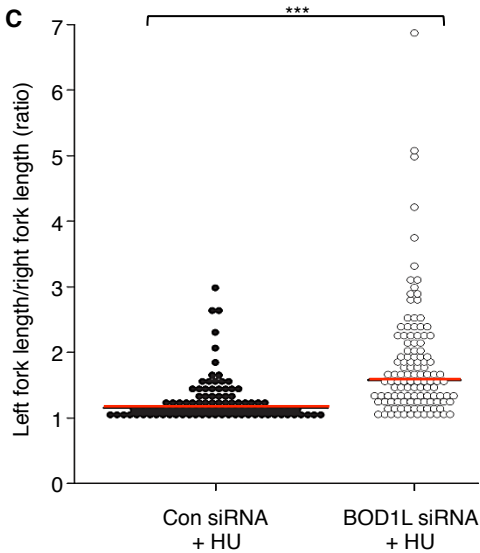


Figure 4

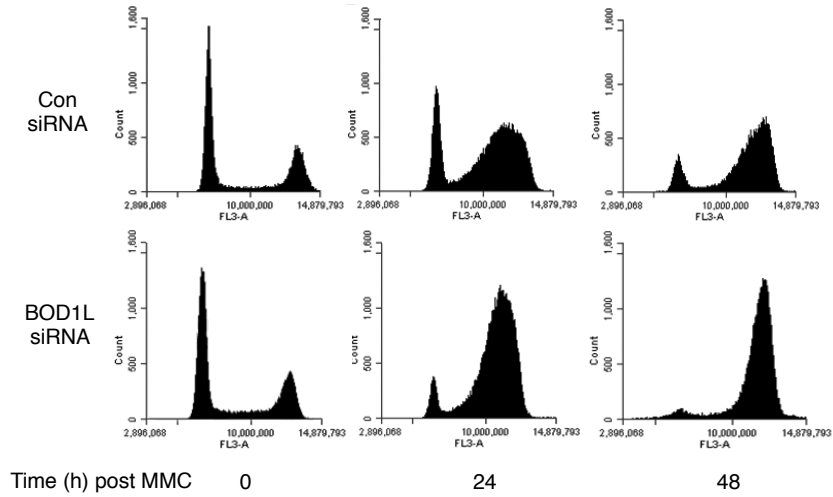
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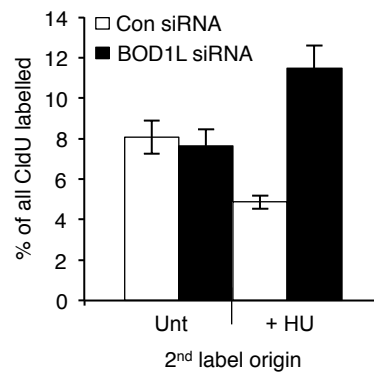
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B



D



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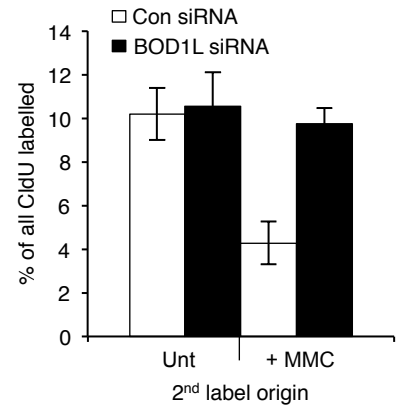
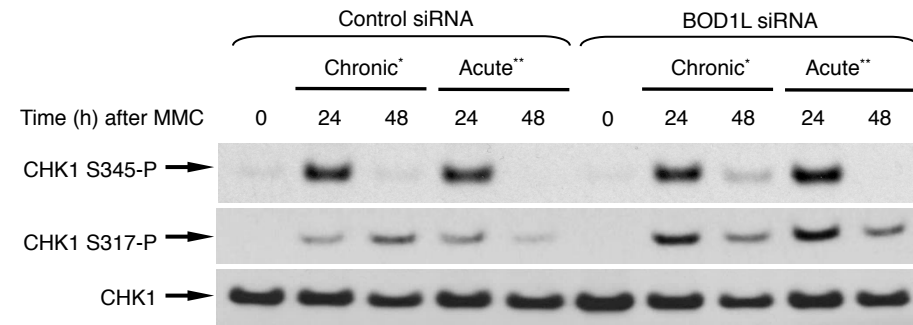
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Figure 5

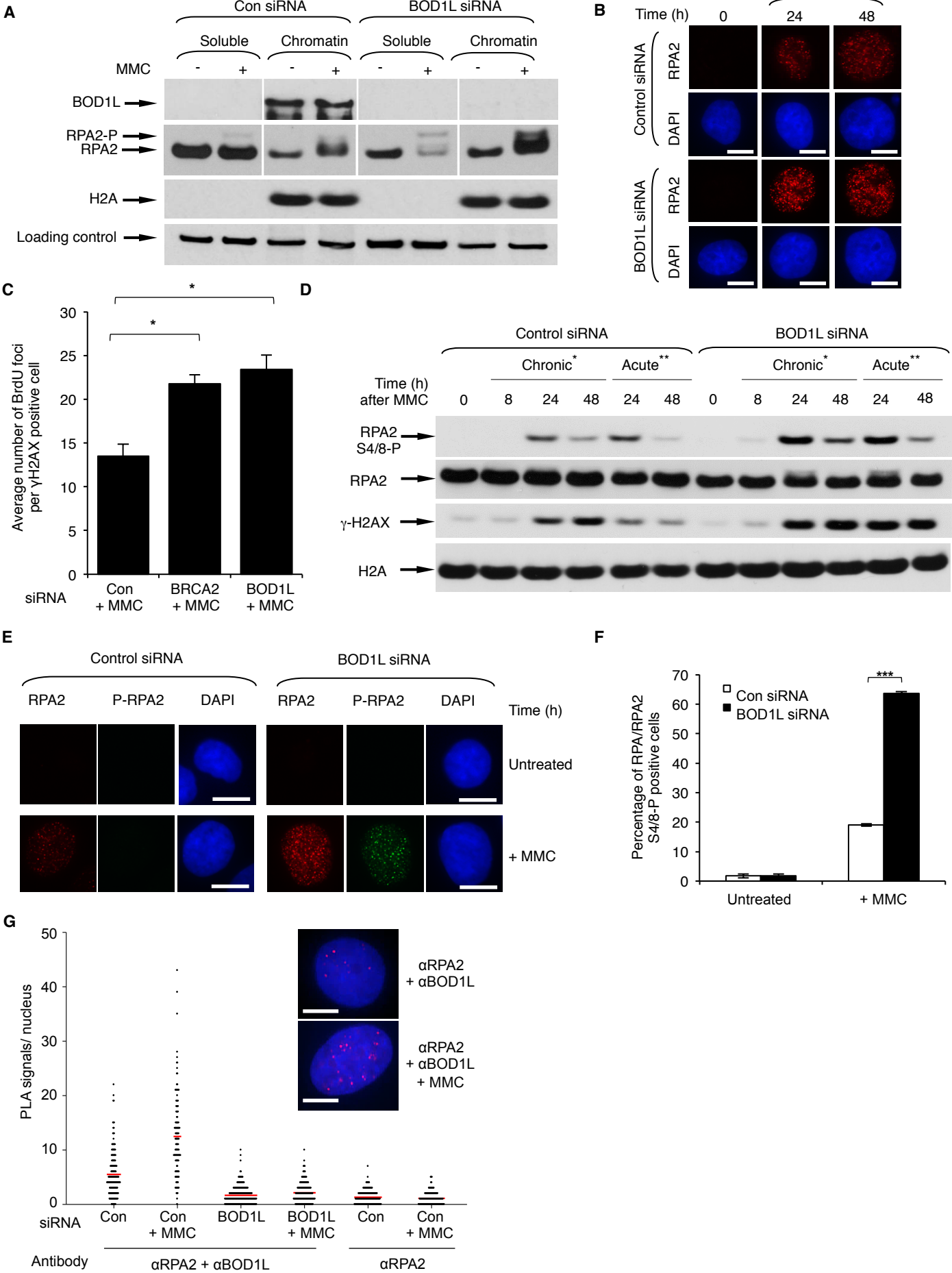


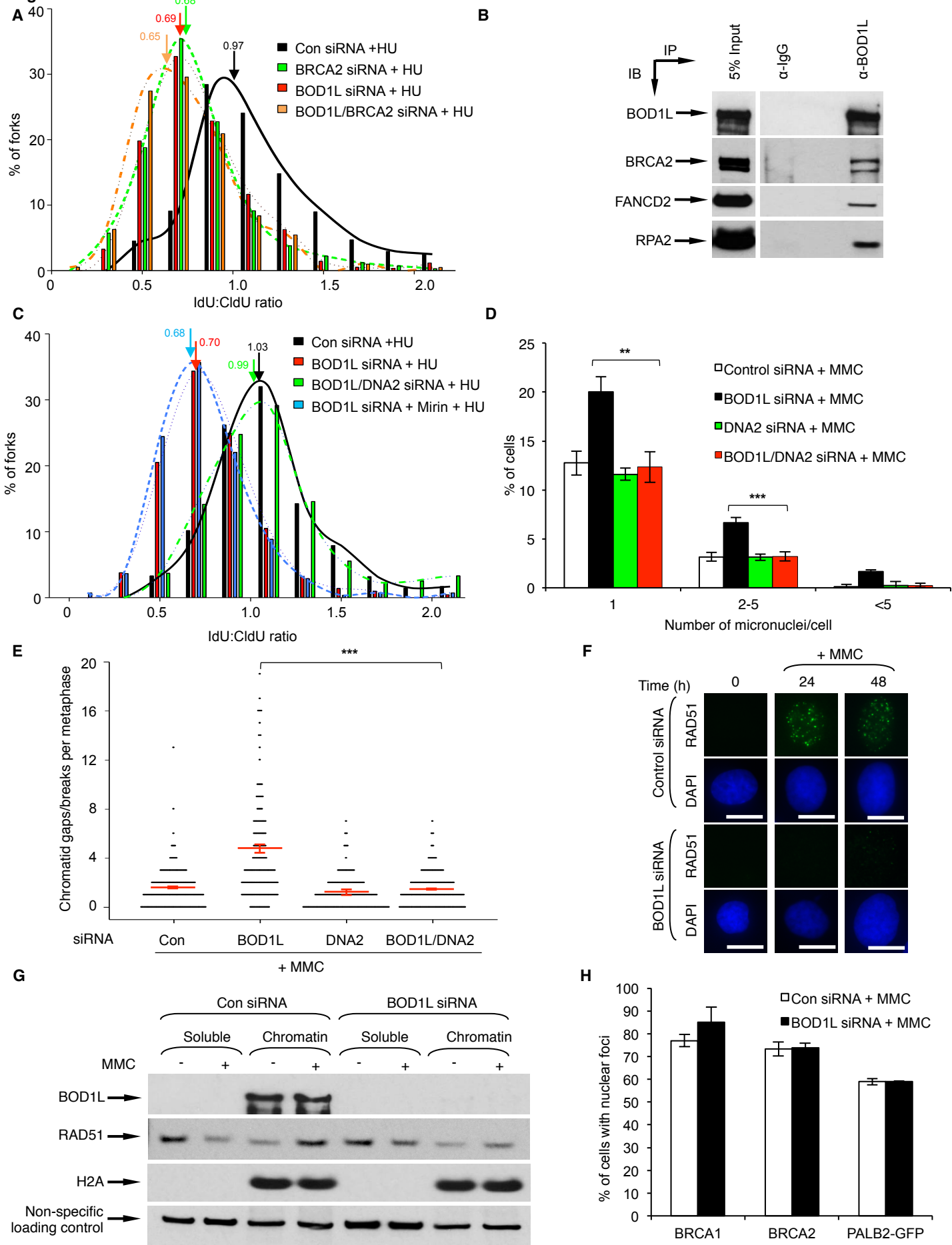
Figure 6

Figure 7

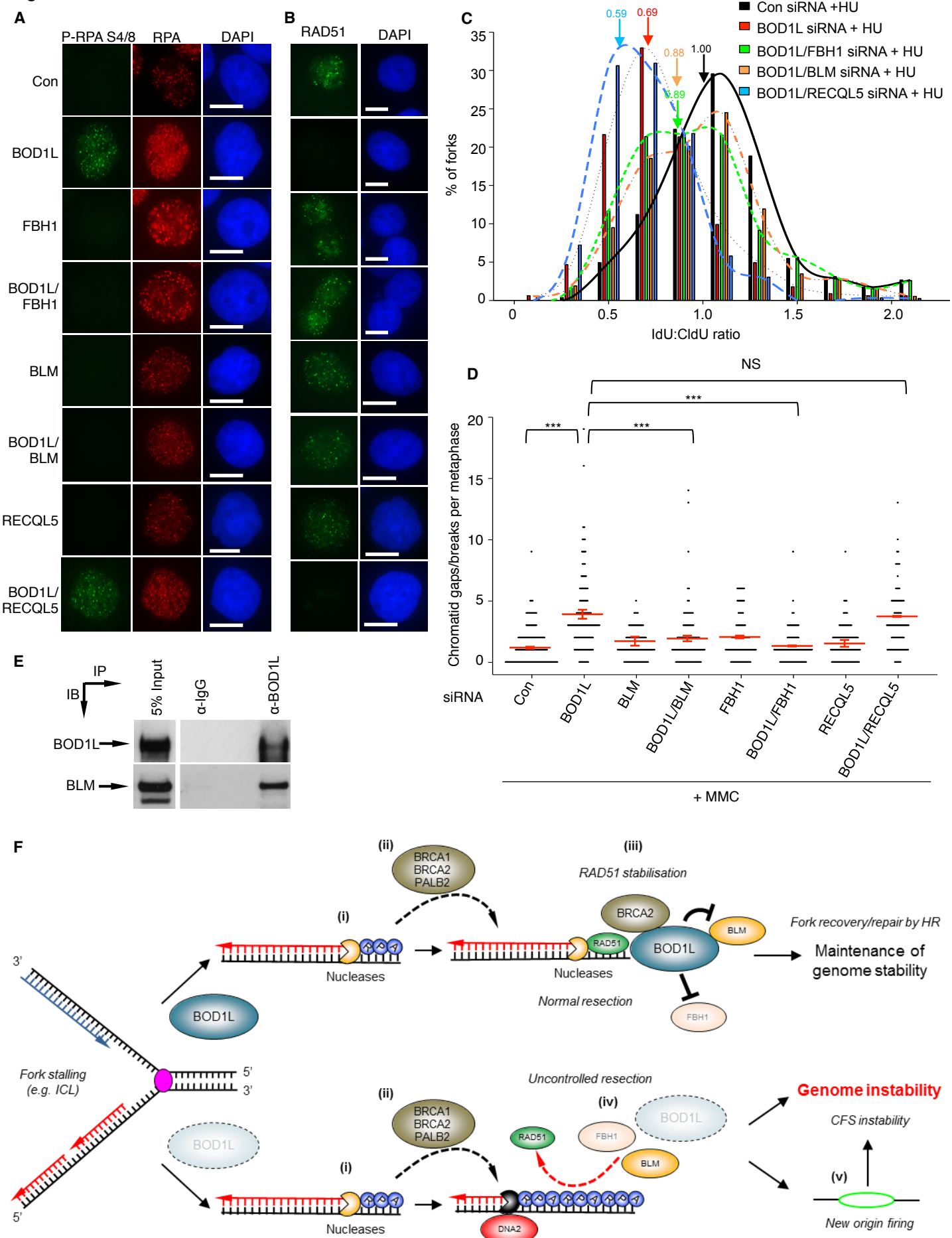


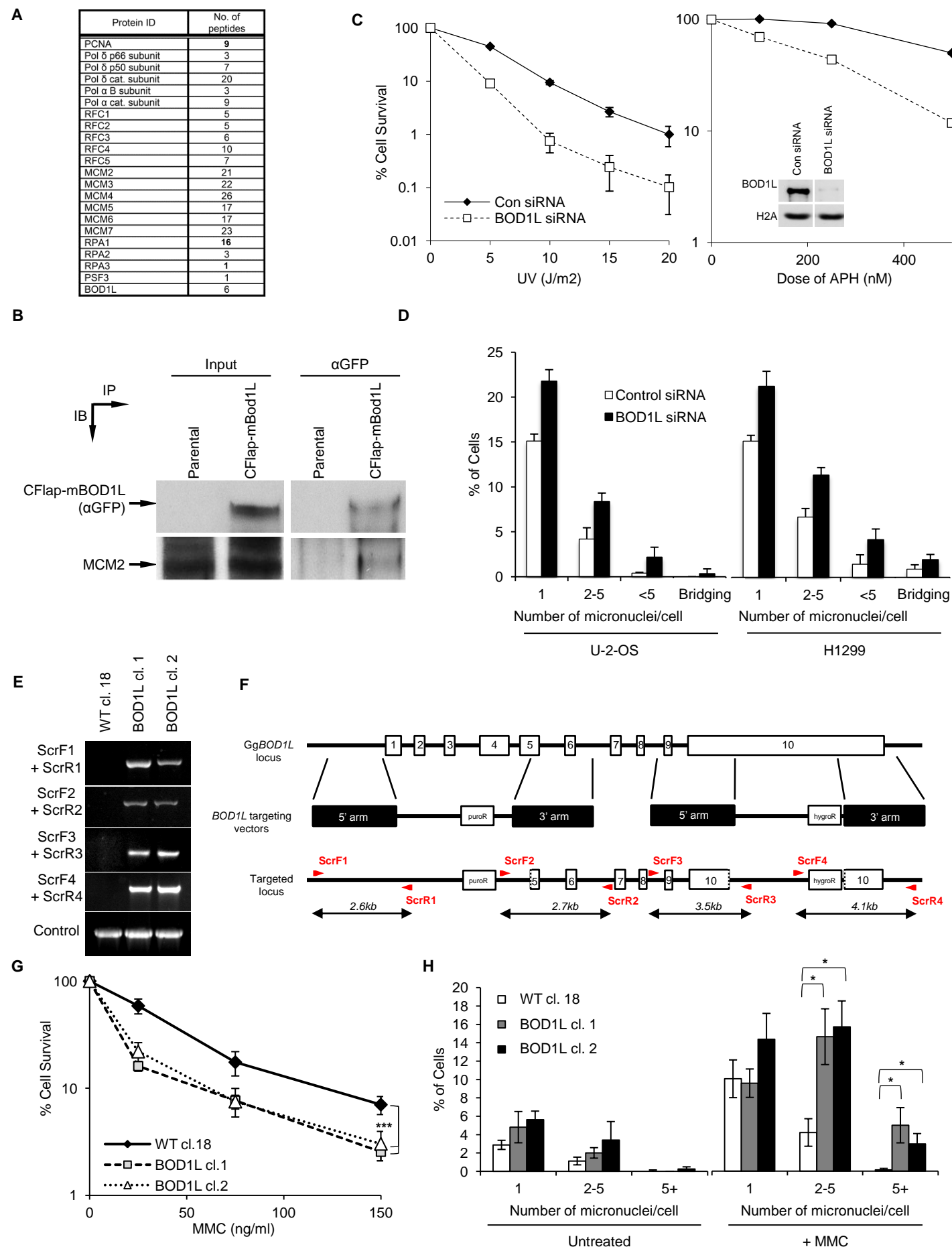
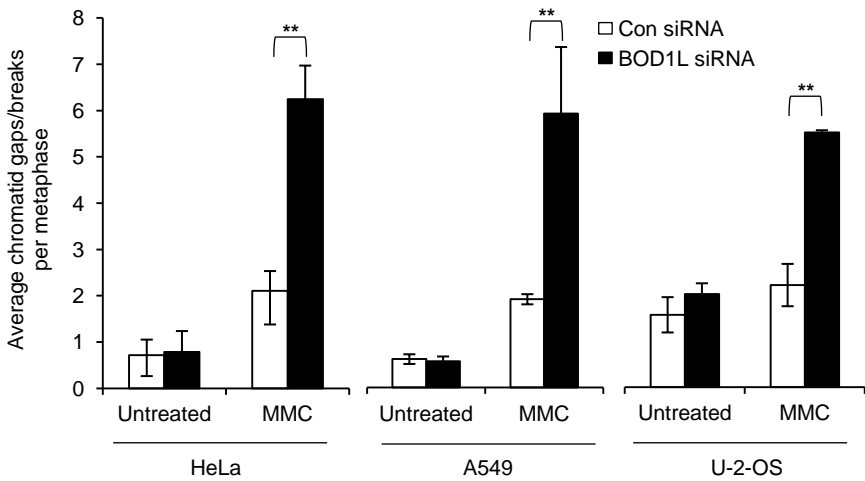
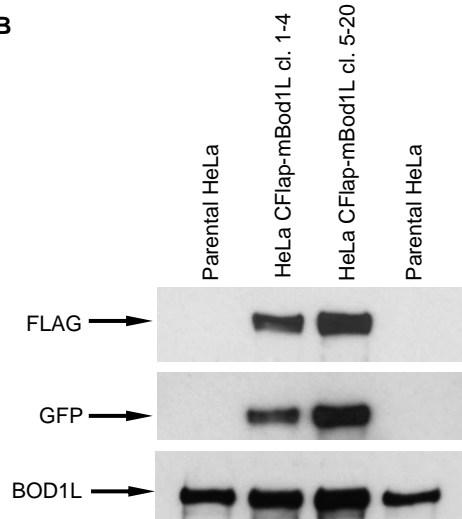
Figure S1

Figure S2

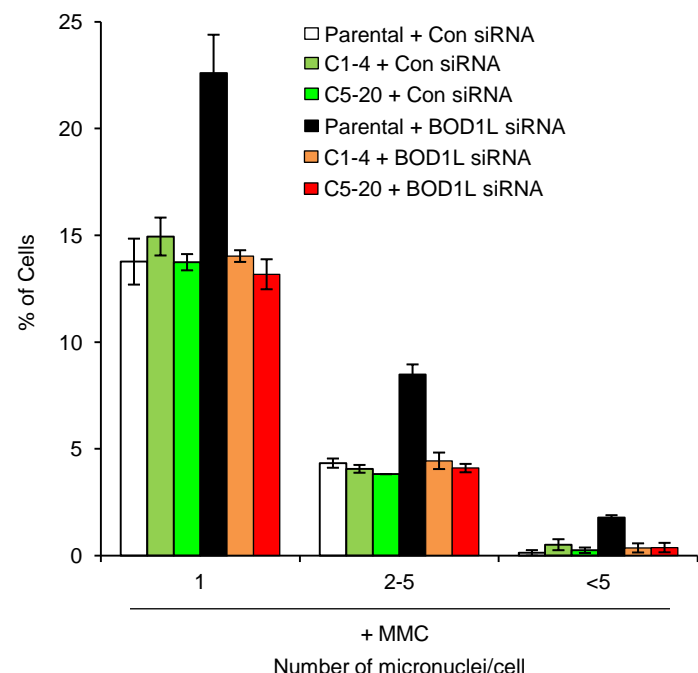
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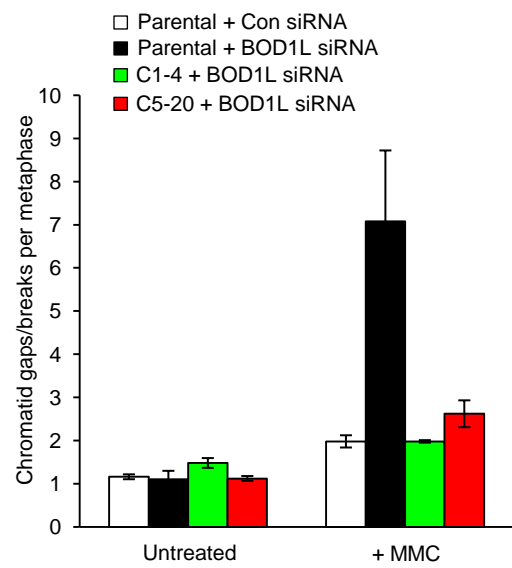
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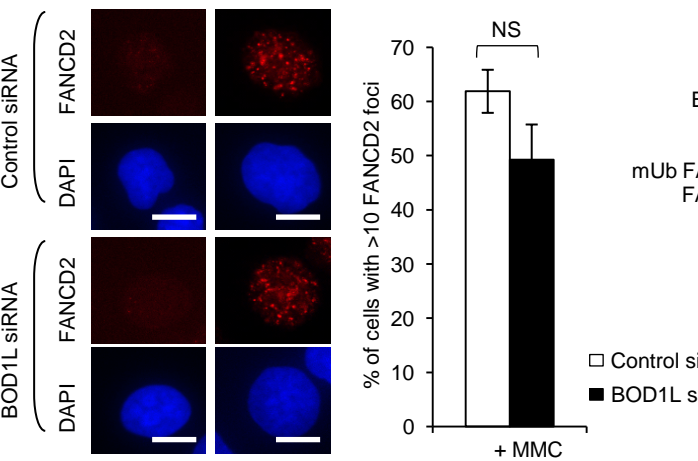
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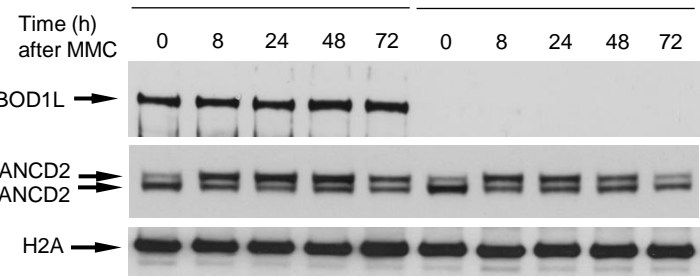


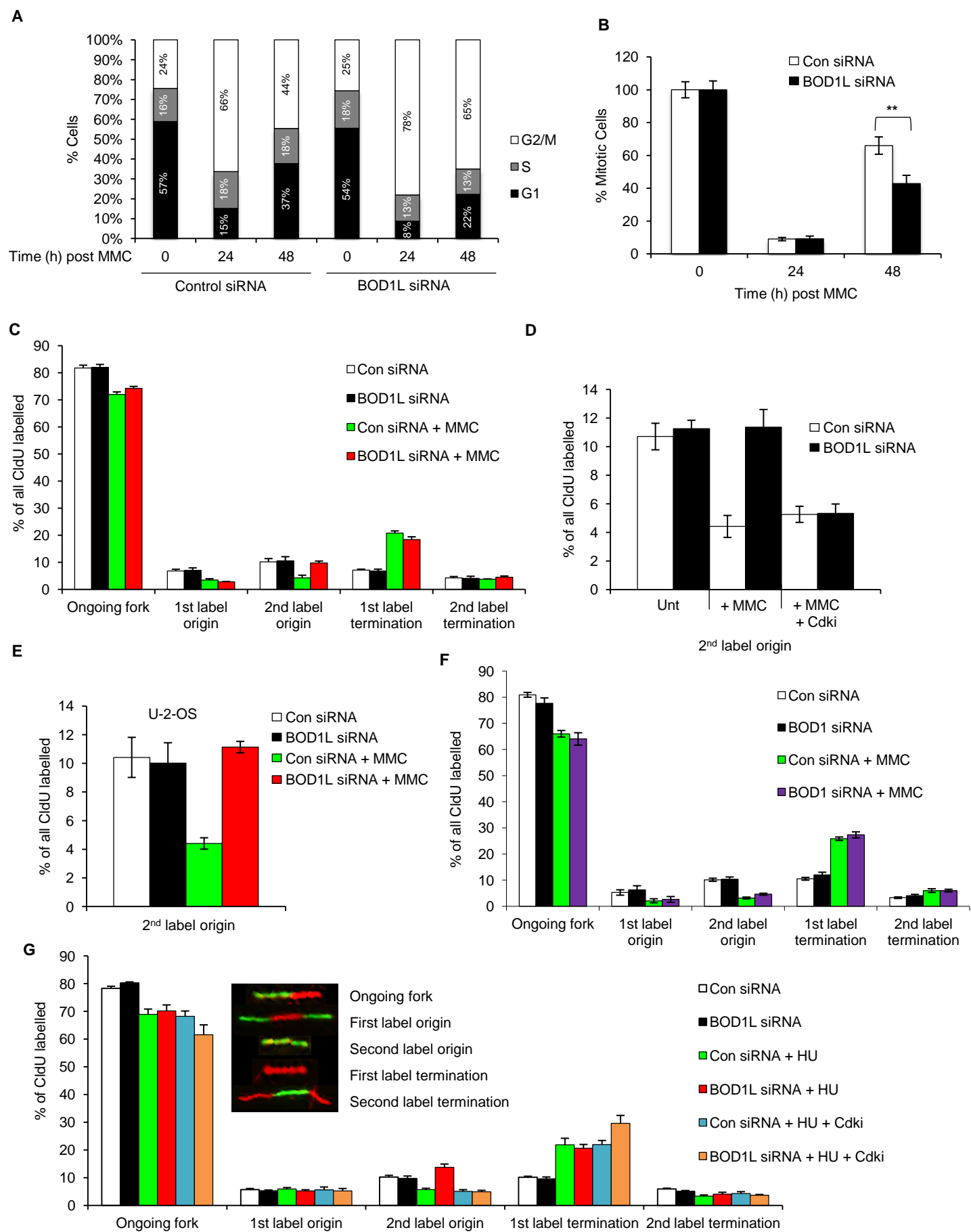
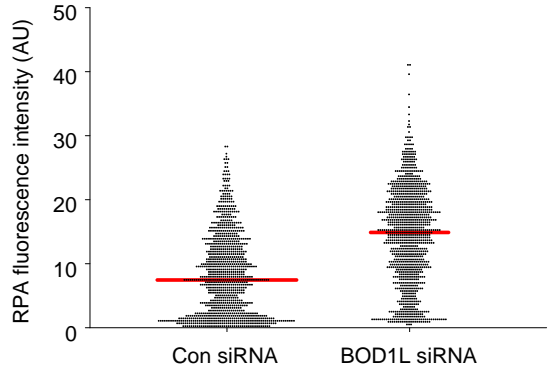
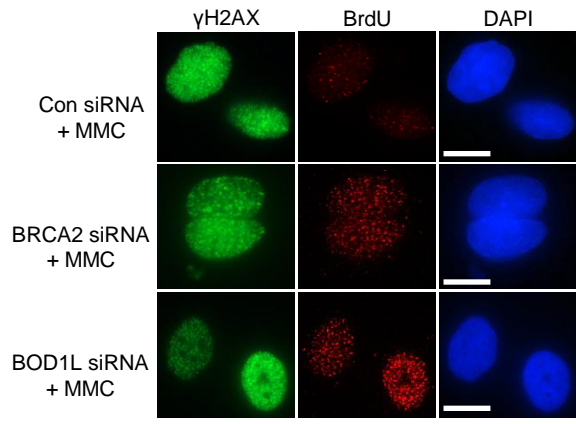
Figure S3

Figure S4

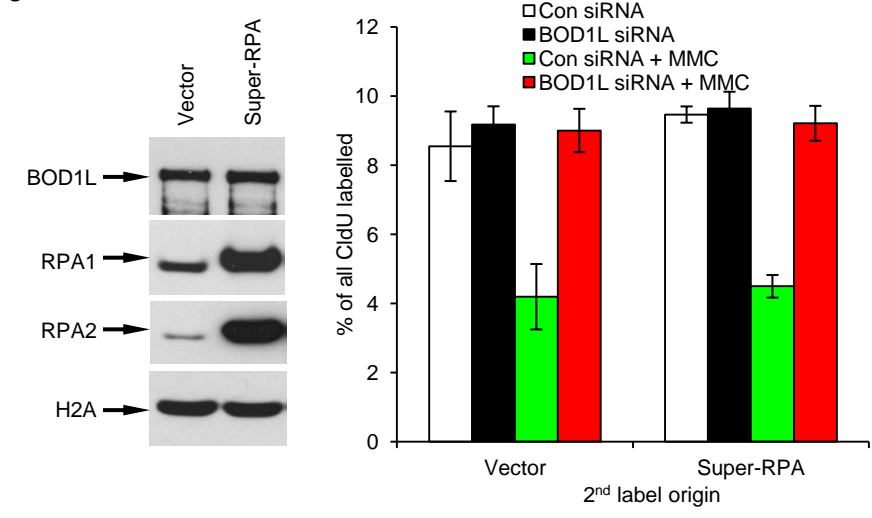
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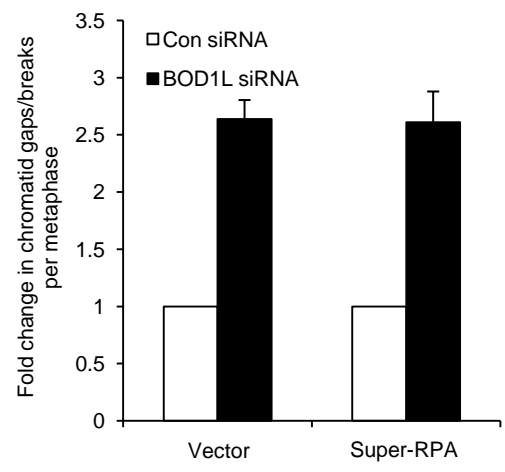
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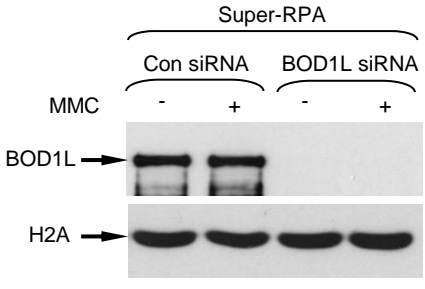
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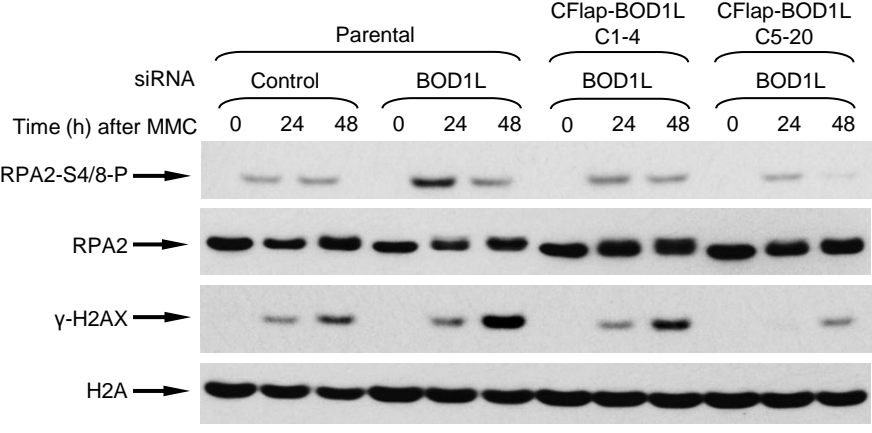
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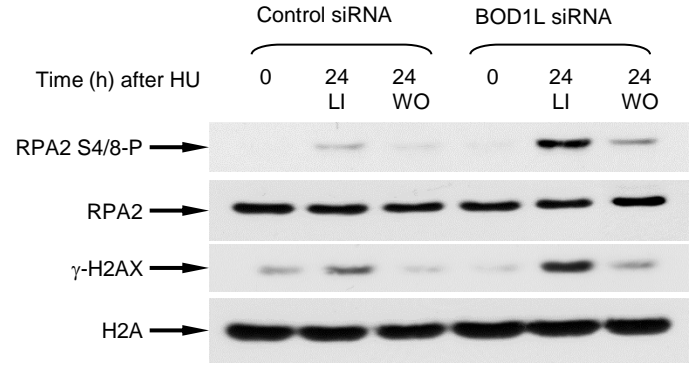


Figure S5

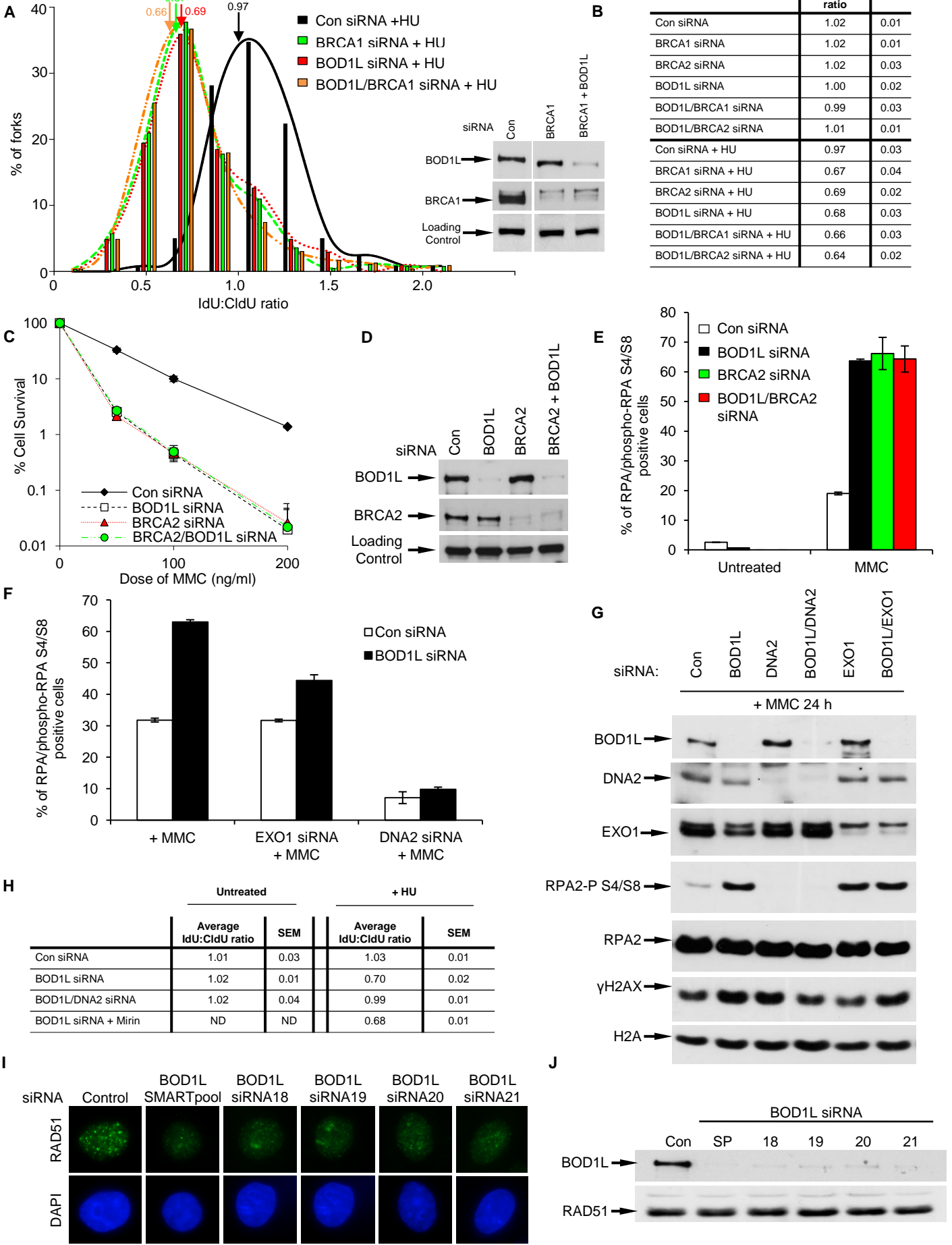


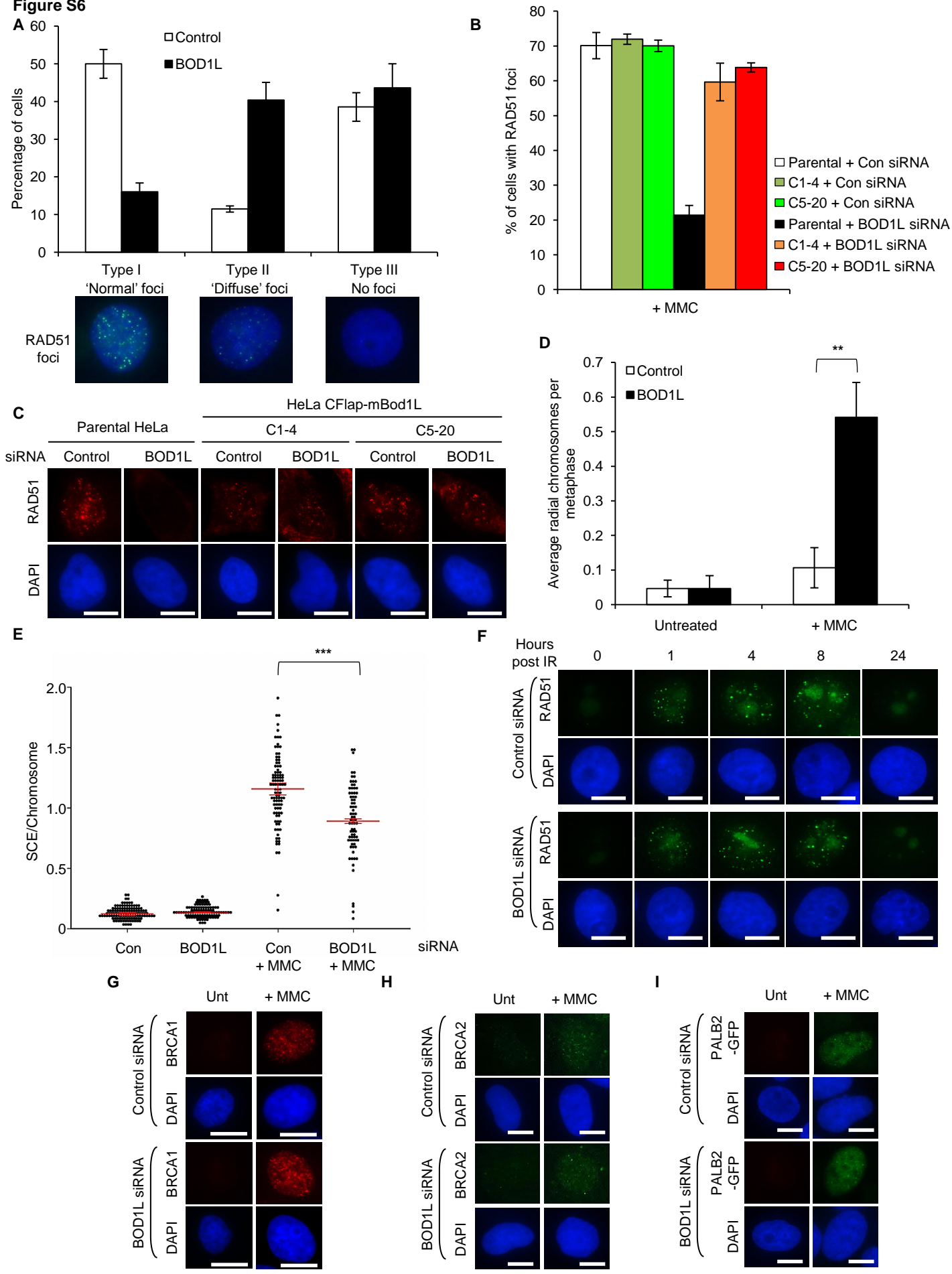
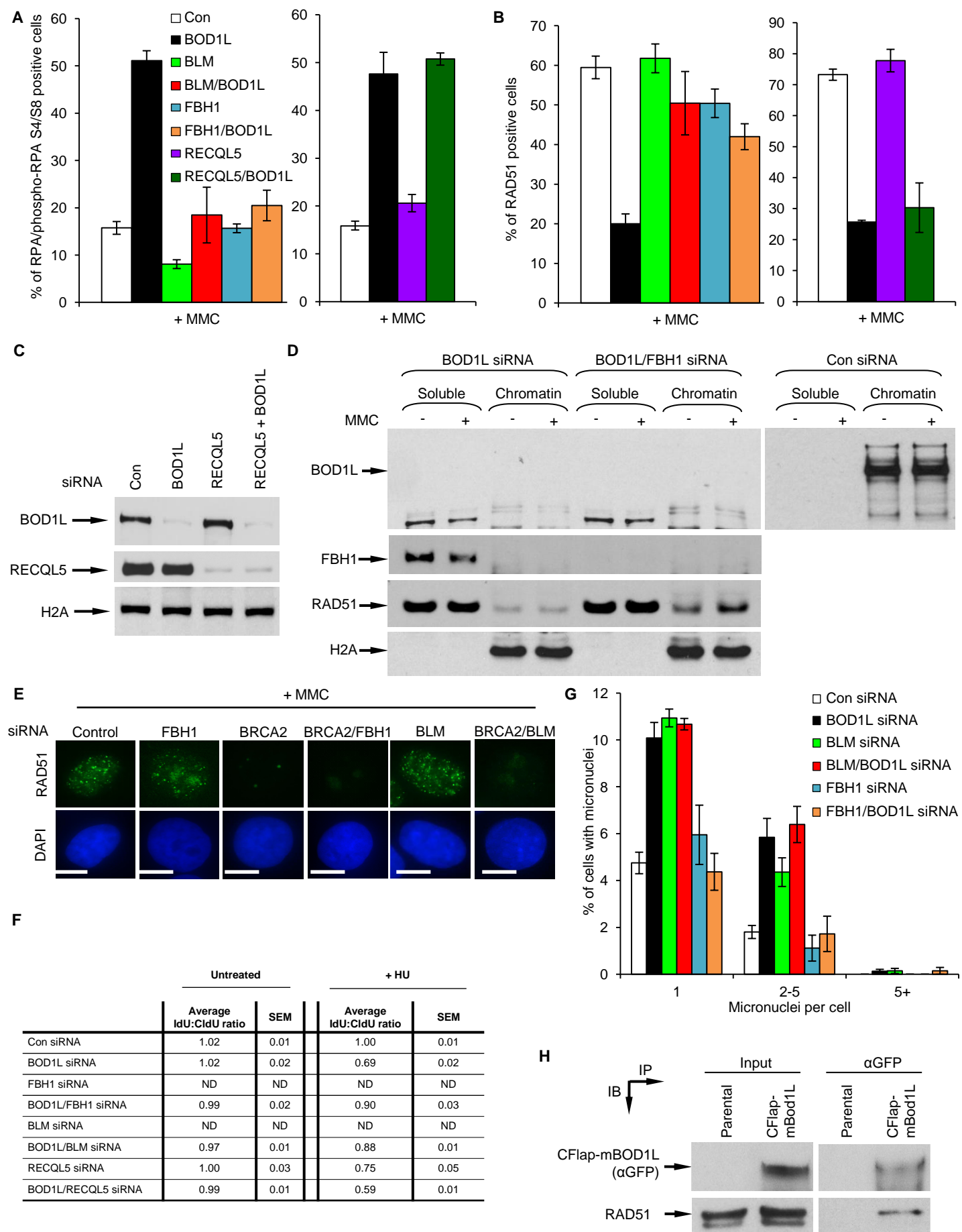
Figure S6

Figure S7



SUPPLEMENTAL FIGURE LEGENDS

Figure S1: BOD1L ensures cellular viability and genome integrity after replication

stress. Related to Figure 1. (A) Total peptides identified by mass spectroscopy analysis of EdU-coprecipitates isolated from HeLa S3 cells. A complete mass spectrometry data set is available on request to the Corresponding Author. **(B)** WCE from parental HeLa or HeLa-CFlap-mBod1L cells (clone 5-20; see also **Figure S2B**) were subjected to IP with anti-GFP antibody, and inputs and recovered immunoprecipitates were analyzed by immunoblotting. Blots originate from a single gel. A white line denotes removal of irrelevant lanes. **(C)** HeLa cells were transfected with the indicated siRNA for 72 h and exposed to the indicated doses of UV-C or aphidicolin (APH), left to form colonies for 14 days, and then stained with methylene blue and counted. **(D)** U-2-OS and H1299 cells were transfected as in (C), exposed to 50 ng/ml MMC for 24 h, and micronuclei formation was assessed by immunofluorescence. **(E)** PCR screening of WT and BOD1L-deleted (Δ exon 1/ Δ exon 10) DT40 clones using the primers in (F). The presence of PCR products denotes successful recombination of the homology arms, and thus deletion of the desired region. **(F)** Schematic of the *Gallus gallus* BOD1L locus (*upper*) with targeting vectors spanning exons 1-5 and exon 10 (*middle*), and a schematic of the targeted Δ exon 1/ Δ exon 10 locus (*lower*). Positions of screening primers are shown. **(G)** WT DT40s, and two clones lacking exons 1-5 and exon 10 of BOD1L, were exposed to the indicated doses of MMC, left to form colonies in soft agar, stained with methylene blue and counted. Plots represent mean data from four independent experiments; error bars represent SEM. Two-way ANOVA. **(H)** WT or BOD1L-deleted DT40s were exposed to 12.5 ng/ml MMC for 24 h, and micronuclei formation assessed by immunofluorescence. Plots represent mean data from three independent experiments; error bars represent SEM; Student's t-test. * = $p < 0.05$; *** = $p < 0.001$.

Figure S2: BOD1L knockdown increases genomic instability after replication stress.

Related to Figure 3. (A) HeLa, A549 and U-2-OS cells were treated as in **Figure 3G**, and damage to metaphase chromosomes was analysed by Geimsa staining and light microscopy.

Graphs integrate data from 50 cells for each condition from three independent experiments. **(B-D)** Parental HeLa or HeLa-CFlap-mBod1L cells (clones C1-4 and C5-20) were transfected with the indicated siRNA for 72 h, and: (B) Whole cell extracts of cells (WCE) were analysed by immunoblotting; (C) Micronuclei formation was assessed by immunofluorescence; (D) Metaphase chromosomes were analysed by Geimsa staining and light microscopy. **(E)** HeLa cells from **Figure 3A** were immunostained with antibodies to FANCD2, and foci formation analysed by fluorescence microscopy. Plot indicates quantification of cells with more than 10 FANCD2 foci per cell from four independent experiments. **(F)** U-2-OS cells were transfected with the indicated siRNA for 72 h, exposed to 250 ng/ml MMC for 3 h, left to recover for the indicated times, and WCE were analysed by immunoblotting. Scale bars = 10 μ m. NS = $p>0.05$; ** = $p<0.01$; Students' t-test.

Figure S3: The effect of BOD1L depletion on DNA replication kinetics. Related to Figure 4. **(A)** Quantification of cell cycle profiles shown in **Figure 4B**. Data is representative of the mean \pm SEM of four independent experiments. **(B)** Cells from (A) were immunostained with antibodies to phosphorylated histone H3-Ser10, and the percentage of mitotic cells determined by flow cytometry. Data represent mean \pm SEM of four independent experiments. **(C)** DNA fibres from **Figure 4E** were quantified. The percentages of ongoing forks, first-label (bidirectional) origins, new origins (IdU-labelled only), first-label terminations (CldU-labelled only) and second label terminations are displayed. **(D)** HeLa cells were treated as in **Figure 4E** except that, where indicated, they were treated with CDK1/2 inhibitor for 3 h prior to pulse labelling. Plot displays percentage of new origins (IdU-labelled only). **(E)** U-2-OS cells were transfected with the indicated siRNA for 72 h, and DNA fibres prepared as in **Figure 4E**. The percentage of new origins (IdU-labelled only) is displayed. **(F)** HeLa cells were transfected with the indicated siRNAs, and the DNA combed and analysed as above. **(G)** Cells were treated as in **Figure 4D**, except that, where indicated, they were treated with CDK1/2 inhibitor for the duration of HU exposure (2 h). Plots display average percentages of the relevant fork

structure(s) from three independent experiments; error bars represent SEM. ** = $p < 0.01$; Students' t-test.

Figure S4: BOD1L is necessary to prevent excessive ssDNA formation and RPA2 hyperphosphorylation after replication stress. Related to Figure 5. (A) Foci formation in HeLa cells from Figure 5B was analysed by fluorescence microscopy, and fluorescence intensity per nucleus was quantified using ImageJ. Lines denote mean values from three independent experiments. (B) U-2-OS cells were treated as in Figure 5C, and immunostained with antibodies to BrdU and γ H2AX. Representative images are shown. Scale bars = 10 μ m. (C-E) Vector U-2-OS or SUPER-RPA U-2-OS cells were transfected with the indicated siRNAs and exposed to 50 ng/ml MMC for 24 h. (C) *Left panel*: WCE of the indicated cell lines were analysed by immunoblotting. *Right panel*: Transfected Vector or SUPER-RPA cells were treated as in Figure 4E, and the plots display the percentage of new origins (IdU-labelled only). (D) Damage to metaphase chromosomes was analysed by Geimsa staining and light microscopy. Graphs integrate data from 150 cells, in total, for each condition from three independent experiments, and are displayed as fold change compared to control siRNA-transfected cells. (E) WCE of the indicated cell lines were analysed by immunoblotting. (F) Parental HeLa or HeLa-CFlap-mBod1L cells (clones C1-4 and C5-20) were transfected with the indicated siRNA for 72 h, exposed to 50 ng/ml MMC for 24 or 48 h, and WCE were analysed by immunoblotting. (G) HeLa cells were transfected with the indicated siRNA for 72 h, exposed to 250 μ M HU for 24 h, and harvested immediately (LI), or left to recover for a further 24 h (WO). WCE were analysed by immunoblotting.

Figure S5: BOD1L is required to suppress aberrant end resection at replication forks after replication stress. Related to Figure 6. (A) U-2-OS cells were transfected with the indicated siRNAs for 72 h, pulsed for 20 min each with CldU and IdU, and exposed to 4 mM HU for 5 h. DNA was visualised with antibodies to CldU and IdU, and replication fork length was calculated. Plots denote the average ratios of IdU:CldU label lengths from three

independent experiments. Arrows indicate mean ratios. (*Inset*) WCE of cells were analysed by immunoblotting. Control siRNA panel is from the same gel as in **Figure S7C**. A white line denotes removal of irrelevant lanes. **(B)** Average values of IdU:CldU label lengths from DNA isolated from cells in **Figures 6A and S6A** is indicated, and SEM are denoted. **(C)** HeLa cells were transfected for 72 h with the indicated siRNAs, exposed to the indicated doses of MMC, left to form colonies for 14 days, and then stained with methylene blue and counted. Plots denote the average values from three independent experiments. **(D)** WCE of cells from (C) were analysed by immunoblotting. Loading control denotes a non-specific protein detected by anti-BOD1L antibody. **(E)** HeLa cells from (C) were exposed to 50 ng/ml MMC for 24 h, immunostained with antibodies to RPA2 and phospho-RPA S4/S8, and foci formation was analysed by fluorescence microscopy. The average percentage of double-positive cells is shown. **(F)** HeLa cells were transfected with the indicated siRNA for 72 h, exposed to 50 ng/ml MMC for 24 h, immunostained with antibodies to RPA2 and phospho-RPA S4/S8, and foci formation was analysed by fluorescence microscopy. The average percentage of double-positive cells from three independent experiments is shown. **(G)** WCE of HeLa cells from (F) were analysed by immunoblotting. **(H)** DNA from U-2-OS cells in **Figure 6C** was visualised with antibodies to CldU and IdU, and replication fork length was calculated. Average values and SEM are denoted. **(I-J)** HeLa cells were transfected with the indicated siRNAs, exposed to 50 ng/ml MMC for 24 h, and either: RAD51 foci formation was analysed by fluorescence microscopy (I); or RAD51 expression was analysed by immunoblotting (J). siRNAs18-21 were individual siRNAs from the SMARTpool.

Figure S6: BOD1L enables RAD51 chromatin loading to promote efficient homologous recombination. Related to Figure 6. **(A)** HeLa cells were transfected with the indicated siRNA for 72 h, exposed to 50 ng/ml MMC for 24 h, and immunostained with antibodies to RAD51. Foci formation was analysed by fluorescence microscopy, and quantified into three distinct phenotypes (representative images are shown in the lower panel). **(B-C)** Parental HeLa or HeLa-CFlap-mBod1L cells (clones C1-4 and C5-20) were transfected with the

indicated siRNA for 72 h, exposed to 50 ng/ml MMC, and immunostained with antibodies to RAD51. (B) Foci formation was analysed by fluorescence microscopy and quantified. (C) Representative images are shown. (D) The incidence of radial chromosome formation was analysed from metaphase spreads prepared as described in **Figure 3G**. ** = $p < 0.01$; Students' t-test. (E) HeLa cells were transfected with the indicated siRNA for 24 h, labelled with BrdU for a further 24 h, and exposed to 25 ng/ml MMC for a further 24 h. Sister chromatid exchanges were quantified from at least 50 cells from three independent metaphase spreads. Line = mean number of SCEs/chromosome. Error bars = SEM. *** = $p < 0.001$; Mann-Whitney ranked sum test. (F) HeLa cells were transfected with the indicated siRNA for 72 h, exposed to 5 Gy of γ -irradiation, and immunostained with antibodies to RAD51. Scale bars = 10 μ m. (G-I) The prevalence of nuclear foci of BRCA1, BRCA2, and PALB2-GFP in HeLa or U-2-OS-PALB2-GFP cells from **Figure 6H**. Foci formation was analysed by fluorescence microscopy, and representative images are shown.

Figure S7: BOD1L acts to restrain anti-recombinase activity to stabilise RAD51 chromatin loading and prevent excessive resection of replication forks. Related to Figure 7. (A-B) HeLa cells were treated as in **Figure 7A/B**, and RPA2/RPA2-PS4/8 foci formation (A) or RAD51 foci formation (B) was analysed by fluorescence microscopy. The average percentage of double-positive cells (A) or RAD51-positive cells (B) is shown. (C) WCE of cells from (A) were analysed by immunoblotting. (D) U-2-OS cells were transfected with the indicated siRNAs, exposed to 100 ng/ml MMC for 24 h, and then fractionated. Soluble and chromatin fractions were analysed by immunoblotting. Blots originate from a single gel. A white line denotes removal of irrelevant lanes. (E) HeLa cells were transfected with the indicated siRNA for 72 h, exposed to 50 ng/ml MMC for 24h, and immunostained with antibodies to RAD51. Scale bars = 10 μ m. (F) U-2-OS cells were treated as in **Figure 7C**, and IdU:CldU ratios were calculated in the presence/absence of HU. Average values and SEM are denoted. (G) Micronuclei formation was assessed by immunofluorescence in HeLa cells from **Figure 7A**. (H) WCE from parental HeLa or HeLa-CFlap-mBod1L cells from **Figure S1B** were

140 subjected to IP with anti-GFP antibody, and inputs and recovered immunoprecipitates were
141 analyzed by immunoblotting. The upper panel is identical to that shown in **Figure S1B**. A white
142 line denotes removal of irrelevant lanes.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell lines

A549, HeLa, HeLa S3, U-2-OS, and H1299 were sourced from the ATCC. U2OS-PALB2-GFP were obtained from F. Esashi. HeLa-FUCCI were obtained from RIKEN BRC.

Drugs and inhibitors

HU, Aphidicolin, and MMC were from Sigma Aldrich, and were used as indicated in the Figure Legends. Cdk1/2 inhibitor III was used at 25 μ M (Merck). dNTP analogues BrdU, EdU, CldU and IdU were from Sigma Aldrich, and were used as indicated. Mirin (Calbiochem) was used at 50 μ M.

Generation of HeLa-CFlap-BOD1L cells.

A BAC containing the full length *Mus musculus* BOD1L locus was obtained from BacPac Resources. This BAC was modified to insert a C-terminal Flap tag by Red/ET recombination following a modified protocol from Genebridges. To generate HeLa-CFlap-BOD1L cells, HeLa cells have been transfected with CFlap-Bod1L BAC using Lipofectamine 2000 (Invitrogen), then selected for 3 weeks with Geneticin and single cell-sorted by FACS following high level of GFP expression. Two clones (C1-4 and C5-20) were expanded and used for further experiments.

Generation of BOD1L knock out DT40 cell lines.

BOD1L knock out DT40 cell lines were generated using two disruption cassettes targeting exons 1-5 and targeting exon 10 of the chicken BOD1L locus. The chicken BOD1L locus was identified by BLAST search using the human protein sequence against the ENSEMBL draft chicken genome sequence. An ENSEMBL predicted transcript encompassed the entire BOD1L gene. From this sequence, two disruption cassettes targeting exons 1-5 and targeting exon 10 were designed. PCR oligos used to amplify 5' and 3' arms of the first construct targeting exons 1-5 were

171 GCGGCCGCGGTCTCGGATCCATGGAGCGACAATGATGACACAGATGG/
 172 GGTGATATCGGCGGCAAGCTGGCTACAGCGTGTTAGGAGGGTTGAGTG and
 173 ATTATACGAACGGTACTCGATGATTTGAAGAGGAAAGTGAAGAAGAACCTGTG/
 174 GGATCCGAGACCGCGGCCGCCCTATCTTACTCACCACCCCAAGTCCTCA respectively.
 175 PCR oligos used to amplify 5' and 3' arms of the second construct targeting exon 10 were
 176 AATATAAAGCTTGCGGCCGCCAGCGTTGTCCAAAGGACATCTG/
 177 GTCAAGCTTCTATTTGGCATCTGTGGCTTGGACTG and
 178 GTACTTGAGTAGCGTGTAATCAGTGCAAGTGCTGATG/
 179 GGCAAGCTTATAGCAGGGTGGGTTGGAAGTAGATG respectively. Targeting constructs
 180 were generated by cloning the PCR products into the pSH vector containing either puromycin
 181 or hygromycin resistance. Transfections and selection of targeted DT40 clones were carried
 182 out as described previously (Niedzwiedz *et al.*, 2004). To confirm the appropriate disruptions
 183 of the GgBOD1L locus, genomic DNA was obtained and the following PCR oligos were used
 184 to screen the clones: ScrF1, TGCATCAGGGATGCACATTCTC; ScrR1,
 185 TAAGACTGCTGCTGACACCTTCAC; ScrF2, GCGGGACTATGGTTGCTGACTAATTGAG;
 186 ScrR2, ACTAGCTGCGTCCCAAAGAGTTTC; ScrF3,
 187 GCTGGCATGCTGGAATGTACTTTATGG; ScrR3,
 188 CTTACAGAGGCGAGTAACTTCCTGTAAC; ScrF4,
 189 ACGATTCCGAAGCCCAACCTTTCATAG; ScrR4, ATCTTTGGAGATGTTCAAGGCCAGGTC
 190 **(Figure S1E).**

191

192 **siRNA Transfections**

193 siRNAs were from Dharmacon as SMARTpool (SP) or individual siRNAs deconvolved from the
 194 SMARTpool: BOD1L (SP, siRNA-18,-19,-20,-21); BOD1 (SP). SP and BOD1L siRNA-19 were
 195 used for all experiments unless stated. siRNA transfections were performed with siRNA
 196 duplexes (100 nM) using Oligofectamine (Invitrogen). Whenever siRNAs were combined, the
 197 total concentration was kept at 100 nM. A custom siRNA targeting lacZ
 198 (CGUACGCGGAUACUUCGAdTdT) was used as a scrambled, non-targeting siRNA, and is

abbreviated as “Control siRNA”, or “Con siRNA”. All experiments were performed 72 h post knockdown unless otherwise stated.

Colony survival assays

Colony survival assays using HeLa cells were carried out as described (Stewart *et al.*, 2003). For colony survival assays with DT40 cells, MMC-treated cells were plated in methylcellulose after exposure to a range of concentrations of the drug. Viable colonies were scored after 2-3 weeks.

DNA combing

DNA combing was carried out essentially as described previously (Petermann *et al.*, 2010). HU (2 mM) or MMC (50 ng/ml) treatments were for 2 h or 24 h respectively. For resection experiments, cells were pulse-labelled with CldU and IdU for 20 min each before a 5 h exposure to 4 mM HU. For quantification of replication structures, at least 250 structures were counted per experiment. The lengths of red or green labelled tracts were measured using ImageJ (National Institutes of Health; <http://rsbweb.nih.gov/ij/>) and arbitrary length values were converted into micrometers using the scale bars created by the microscope.

Chromatin fractionation

Subcellular fractionations were performed in U-2-OS cells essentially as described in (Mendez and Stillman, 2000), except that chromatin fractions were washed once after isolation in 200 mM NaCl, 3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, plus protease inhibitor cocktail, and re-suspended in UTB.

Metaphase spreads, SCEs and FISH

Chromosomal aberrations and sister chromatid exchanges (SCEs) were scored in Giemsa stained metaphase spreads. For chromosome aberrations, demecolcine (Sigma) was added 3-4 h prior to harvesting at a final concentration of 0.2 µg/ml. Cells were harvested by

trypsinisation, subjected to hypotonic shock for 1 hour at 37°C in 0.3 M sodium citrate and fixed in 3:1 methanol:acetic acid solution. Cells were dropped onto acetic acid humidified slides, stained for 15 minutes in Giemsa-modified (Sigma) solution (5% v/v in H₂O) and washed in water for 5 minutes.

For SCEs, 10 µM BrdU (Sigma) was added to the medium for two complete cycles (approximately 48 hours) before collection and 25ng/ml MMC was added 24 h before collection. 0.2 µg/ml demecolcine was added 3 h prior to harvesting and metaphase spreads were obtained as described above. Before Giemsa staining, slides were incubated in Hoescht 33258 solution (10 µg/ml) for 20 minutes, exposed to UV light (355 nm) for 1 hour and washed for 1 hour at 60°C in 20× SCC. Cells were harvested as described above.

Fragile site FISH was performed as previously described by (Le Tallec *et al.*, 2011). Probes for the common fragile sites FRA3B and FRA16D were made from BACs RP11-170K19 and RP11-281J9, respectively (Children's Hospital Oakland Research Institute) and were labelled with Biotin-conjugated nucleotides using the BIOPRIME DNA Labelling System (Invitrogen) according to manufacturer's protocol. For dioxigenin incorporation, the BIOPRIME DNA Labelling System was used, but Dioxigenin-conjugated dNTPs (Roche) were used instead of biotin-conjugated dNTPs. Probes were purified using Illustra Probequant G-50 micro columns (GE Healthcare).

Flow cytometry

Flow cytometry was carried out as described previously (Townsend *et al.*, 2009). Briefly, HeLa cells were harvested, fixed in 70% ethanol at -20°C for at least 1 h, and permeabilised with 0.25% Triton-X100 for 15 at 4°C. For immuno-detection of phospho-histone H3 (Ser10), cells were then incubated with primary antibody for 1 h, washed in 1% BSA, and counterstained with Alexa Fluor-488 goat anti-mouse IgG antibody. Cells were then washed twice with 1% BSA, and stained with 25 µg/ml propidium iodide containing 0.1 mg/ml RNase A. Cells were

analysed using an Accuri flow cytometer (BDBiosciences) in conjunction with CFlowplus software. Data represents that obtained from at least 30,000 cells.

Antibodies and Western blotting

Whole cell extracts were obtained by sonication in UTB buffer (8 M Urea, 50 mM Tris, 150 mM β -mercaptoethanol, protease inhibitor cocktail (Roche)) and analysed by SDS-PAGE following standard procedures. The following antibodies were used: H2A, γ -H2AX, BRCA2, RPA2, RAD51 (Merck Millipore); MRE11, phospho-histone H3 Ser-10, phospho-CDK2 Tyr-15 (Cell Signalling); MCM7, CHK1, PCNA, CDK2, FANCD2, BRCA1, BRCA2 (Santa Cruz Biotechnology); RECQL5, FANCA, EXO1, phospho-RPA2 Ser-4/8, phospho-CHK1 Ser-317, phospho-CHK1 Ser-345, MLL1 (Bethyl); CENPA, DNA2, BOD1, PCNT-1 (Abcam), α -tubulin, FLAG (Sigma Aldrich); GFP (Roche); BrdU (CldU) (AbD Serotec); BrdU (IdU) (Becton Dickinson); MCM2 (BD Transduction); 53BP1 (G. S. Stewart); PICH (H. Yu). Affinity purified polyclonal anti-BOD1L antibodies were generated by immunising rabbits with a purified GST-fusion protein spanning amino acids 1,900 to 2,501 of human BOD1L (Accession number: NP_683692.2) (Eurogentec).

Immunoprecipitations

HeLa nuclear cells extracts (Cilbiotech) were clarified by centrifugation at 44,000 x g, immunoprecipitated with 5 μ g of anti-BOD1L antibody or IgG for 3 h at 4 °C. After further clarification, immune complexes were isolated using protein-A sepharose (GE Healthcare), and analysed by immunoblotting.

iPOND

EdU-labeled sample preparation: Logarithmically growing HeLa S3 cells (1×10^6 per ml) were incubated with 10 mM EdU for 10 min. Following EdU labelling, cells were fixed in 1 % formaldehyde, quenched by adding glycine to a final concentration of 0.125 M and washed in PBS three times. Collected cell pellets were frozen at -80 °C and cells were permeabilised by

resuspending in ice cold 0.25 % Triton-X/PBS at a concentration of $1-1.5 \times 10^7$ cells per ml and incubating on ice for 30 min. Before the Click reaction, samples were washed once in 0.5 % BSA/PBS and once in PBS.

Click reaction: Cells were incubated in Click reaction buffer for 1h at room temperature containing 10 μ M azide-PEG(3+3)-S-S-biotin conjugate (Click ChemistryTools, cat. no AZ112-25), 10 mM sodium ascorbate, and 1 mM copper (II) sulfate (CuSO_4) in PBS. The 'no Click' reaction contained DMSO instead of biotin-azide. Following the Click reaction, cells were washed once in 0.5 % BSA/PBS and once in PBS. Cells were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 1 % SDS) containing protease inhibitor cocktail (Roche) and sonicated using a Diagenode Bioruptor® Plus for 40 cycles (30 sec on/30 sec off). Samples were centrifuged at 14,500 xg at 4°C for 30 min and the supernatant was diluted 1:3 with NTN buffer (100 mM NaCl, 20 mM Tris pH 7.4 and 0.05 % NP40) containing protease inhibitors. An aliquot was taken as an input sample.

Purification: Streptavidin-agarose beads (Novagen) were washed three times in NTN buffer containing protease inhibitor cocktail. 200 μ l of bead slurry was used per 1×10^8 cells. The streptavidin-agarose beads were resuspended 1:1 in NTN buffer containing protease inhibitors and added to the samples. Samples were then incubated at 4 °C for 4 h in light exclusion. Following binding, the beads were then washed 4x with 1 ml NTN buffer and protein-DNA complexes were eluted by incubating with 5mM DTT in NTN buffer. Cross-links were reversed by incubated samples in SDS sample buffer at 95 °C for 12 min. Proteins were resolved on SDS-PAGE and detected by immunoblotting, or mass-spectrometry analysis was performed on the eluates. Mass spectrometry was carried out as described previously (Adelman *et al.*, 2013). A complete mass spectrometry data set is available on request to the Corresponding Author.

Microscopy and Image Analysis

HeLa, H1299, U-2-OS, HeLa-FUCCI or A549 cells were grown on glass coverslips. DT40 cells were grown in suspension, and dropped onto poly-L-lysine coated coverslip for 15 min. Cells were washed with PBS twice before fixation. For α -tubulin, PCNT-1, PCNA, CENPA and 53BP1 immuno-detection, cells were fixed with methanol at -20 °C for 10 minutes. For PICH, FANCD2 and EdU detection, and for 53BP1 immuno-detection in FUCCI cells, cells were fixed in 3.6% paraformaldehyde for 10 min at room temperature before permeabilisation with nuclear extraction buffer (10 mM PIPES, 20 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 0.5% Triton X-100) for 10 minutes. For RAD51, γ -H2AX, BRCA2 and RPA immuno-detection, cells were pre-treated with nuclear extraction buffer for 5 minutes on ice, and fixed in 3.6% paraformaldehyde for 10 minutes at room temperature. For in situ detection of nascent DNA in mitotic and interphase cells the Click-iT DNA Alexa Fluor 495 Imaging Kit (Invitrogen) was used. For ssDNA (BrdU) analyses, cells were pre-treated with nuclear extraction buffer for two 5 consecutive minute incubations on ice, then fixed as above. After fixation, cells were washed with PBS three times and then blocked with ADB (Antibody Dilution Buffer; 5% FCS in PBS) for 1 h at 4°C. Cells were incubated with primary antibody (diluted in ADB) for 1 h at room temperature, washed with ADB and then counterstained with Alexa Fluor-488 goat anti-rabbit IgG, Alexa Fluor-594 goat anti-mouse IgG, Alexa Fluor-350 goat anti-rabbit IgG, or Alexa Fluor-555 donkey anti-rabbit IgG secondary antibodies (Molecular Probes) diluted in ADB, for 1 h at room temperature. Cells were then washed twice with ADB and coverslips were mounted onto glass slides with Vectashield mounting agent containing 0.4 μ g/ml DAPI (Vectashield). Fluorescence images were taken using a Nikon E600 Eclipse microscope equipped with a 60X oil lens, and images were acquired and analysed using Volocity Software v4.1 (Improvision). For ssDNA analyses, BrdU foci were enhanced using the ImageJ convolve function, and the number of nuclear foci/cell quantified.

Proximity ligation assays

338 For proximity ligation assays (PLA), cells were fixed/permeabilised as appropriate for the
339 primary antibodies used, incubated in primary antibody, and in situ proximity ligation was
340 performed using Duolink Detection Kit in combination with anti-Mouse PLUS and anti-Rabbit
341 MINUS PLA Probes, according to the manufacturer's instructions (Sigma Aldrich Duolink).
342 Nuclear foci were imaged as above, and the number of nuclear foci/cell quantified using
343 ImageJ.

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